Orthogonal analytical approaches to detect potential contaminants in heparin

Marco Guerrini a,1, Zhenqing Zhang b,1, Zachary Shriver c,1, Annamaria Naggi a, Sayaka Masuko b, Robert Langer c,2, Benito Casu a, Robert J. Linhardt a, and Giangiacomo Torri a, and Ram Sasiskeharan d,2

aIstituto di Richerche Chimiche e Biochimiche “G. Ronzoni,” via Giuseppe Colombo 81, 20133 Milan, Italy; bCenter for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180; and cHarvard-MIT Division of Health Sciences and Technology, Department of Biological Engineering, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139

Contributed by Robert Langer, June 23, 2009 (sent for review June 3, 2009)

Heparin is a widely used anticoagulant and antithrombotic agent. Recently, a contaminant, oversulfated chondroitin sulfate (OSCS), was discovered within heparin preparations. The presence of OSCS within heparin likely led to clinical manifestations, most prevalently, hypotension and abdominal pain leading to the deaths of several dozens of patients. Given the biological effects of OSCS, one continuing item of concern is the ability for existing methods to identify other persulfonated polysaccharide compounds that would also have anticoagulant activity and would likely elicit a similar activation of the contact system. To complete a more extensive analysis of the ability for NMR and capillary electrophoresis (CE) to capture a broader array of potential contaminants within heparin, we completed a systematic study of NMR, both mono- and bidimensional, and CE to detect both various components of sidestream heparin and their persulfonated derivatives. We show that given the complexity of heparin samples, and the requirement to ensure their purity and safety, use of orthogonal analytical techniques is effective at detecting an array of potential contaminants that could be present.

Heparin is a complex pharmaceutical agent, has been used clinically since the early part of the 20th century in the prevention and initial treatment of thrombosis. Heparin is a sulfated glucosaminoglycan comprised of polysaccharide chains of different molecular weights, with a well-defined statistical composition in terms of disaccharide units and pattern of sulfation. Although the chains within a heparin preparation can vary based on length and sulfation pattern, each is comprised of a disaccharide repeat unit of N-sulfation pattern, each is comprised of a disaccharide repeat unit of N-acetyl-D-glucosamine (1, 2).

Pharmaceutical grade heparin has historically been defined based on units of activity using plasma clotting assays (10), rather than on the basis of molecular properties. This is changing because of the heparin contamination crisis and the advent of sophisticated methods for polysaccharide analysis. Various compendia standard-setting organizations across the globe, including the United States Pharmacopeia and the European Pharmacopeia, have proposed implementation of tests, based on defined biochemical assays, NMR spectroscopy and capillary electrophoresis (CE)/high performance liquid chromatography (HPLC), to measure specific structural and charge properties of heparin (10). Reexamination of historical lots of pharmaceutical heparin, prepared since 1941, using these types of methods, suggest a surprising level of purity and structural consistency (11).

Changes in testing are warranted because an oversulfated (OS) chondroitin sulfate (CS) contaminant, associated with anaphylactoid reactions, entered the heparin supply chain in a variety of countries, including the United States (12–14). In late 2007 and early 2008, clusters of serious adverse events, including acute hypotension, were reported in patients undergoing hemodialysis during the purification, are heparan sulfate-like sequences (HS), having a lower anionic charge density and characterized by a ratio of N-acetylgalcosamine to N-sulfoglucosamine of >3.0, a sulfoto-carboxyl ratio of <1.6 and a D-glucuronic to L-iduronic acid ratio of >2 (5). On the other end of the continuum, pharmaceutical heparin has a sulfato-to-carboxyl group ratio of 2.4–2.8 and contains primarily N-sulfoglucosamine and L-iduronic acid, with >50% of its disaccharide units comprised of the trisulfated disaccharide, 3S,4S,6S (6). Between these two ends of the continuum is a diversity of chains with differential N- and O-sulfonation. For example, various heparin/HS fractions can be isolated from crude heparin with distinct structural and functional attributes (5). Several studies have identified methods to separate and characterize various subfractions of heparin, including identification and quantification of slow- and fast-moving components, based on electrophoretic mobility (7–9). These designations, and others, are useful in that they enable the comparison of heparin across different manufacturers and species. For example, fast moving heparin is enriched in the anticoagulant function of heparin and can be used as a screen to assess the anticoagulant activity of heparins isolated from different tissues or organisms (7, 8).

Pharmaceutical grade heparin has historically been defined based on units of activity using plasma clotting assays (10), rather than on the basis of molecular properties. This is changing because of the heparin contamination crisis and the advent of sophisticated methods for polysaccharide analysis. Various compendia standard-setting organizations across the globe, including the United States Pharmacopeia and the European Pharmacopeia, have proposed implementation of tests, based on defined biochemical assays, NMR spectroscopy and capillary electrophoresis (CE)/high performance liquid chromatography (HPLC), to measure specific structural and charge properties of heparin (10). Reexamination of historical lots of pharmaceutical heparin, prepared since 1941, using these types of methods, suggest a surprising level of purity and structural consistency (11).

Changes in testing are warranted because an oversulfated (OS) chondroitin sulfate (CS) contaminant, associated with anaphylactoid reactions, entered the heparin supply chain in a variety of countries, including the United States (12–14). In late 2007 and early 2008, clusters of serious adverse events, including acute hypotension, were reported in patients undergoing hemodialysis...
and receiving heparin. The observed patient reactions were linked to suspect heparin lots containing a then unidentified contaminant that was subsequently determined to be OSCS (structure represented in Fig. S2). The predominant structure of OSCS was found to have 4 sulfates per disaccharide, a disaccharide unit that, to date, has never been found in nature and is structurally distinct from other GAG impurities, such as DS, which may arise from an incomplete purification process. Additionally, the structure of OSCS suggests that it was persulfonated (complete or nearly complete sulfonation of all hydroxyl groups) before its introduction into heparin. Accordingly, the presence of OSCS in heparin raises the concern that other components of the heparin purification process, including DS, HA, or the so-called side-stream products in the manufacture of heparin, could also be present as persulfonated derivatives. In this study, we present a detailed analysis of side-stream products in the manufacture of heparin and identify characteristic signatures that can be used to identify persulfonated derivatives of these components, either alone or as a mixture, when present within pharmaceutical grade heparin. Thus, this study seeks to define the makeup of various components of side-stream heparin, and identify the distinctive features in the NMR/CE analysis of persulfonated derivatives of each polysaccharide component of side-stream heparin.

Results
Composition of Side-Stream Heparin. Starting from crude heparin, a number of chemical and enzymatic treatment steps, precipitations, and other purification/recovery processes are performed to derive purified, pharmaceutical-grade heparin. Previous studies have determined that the heparin purification process largely eliminates a number of related GAGs and other impurities, present within crude heparin (3, 4). Examination of the $^1$H NMR of crude heparin (Fig. S3) indicates that it is a mixture of polysaccharide components, many of which are substantially removed after purification. In addition, the spectrum of crude heparin exhibits broader peaks when compared with that of pharmaceutical heparin; this can be attributed at least in part to the heterogeneity of the sample. To further define molecular changes that occur during the purification of heparin, we used two-dimensional heteronuclear single quantum coherence (HSQC) spectroscopy to examine the compositional makeup of crude heparin compared with purified heparin (Fig. 1).

Inspection of the NMR spectrum focusing on the H-1/C-1 (anomeric) region for crude heparin indicates that it contains a mixture of GAG components, including hyaluronic acid (HA), CS, and DS, and undersulfated heparan sulfate (HS)-like sequences, all of which are substantially removed after purification, as well as heparin. For example, in the HSQC spectrum, the H-1/C-1 signals for the glucuronate are clearly observed for HA (4.47/106.0 ppm), CS A (4.46/106.6), and CS C (4.50/107.1). In addition, the iduronic acid of DS is present at 4.87/106.1 ppm and N-acetylgalactosamine signal (4.67/104.9) is also clearly distinguishable. These signals are largely absent from purified heparin, indicative of the fact that these species are removed as part of the purification procedure.

For the heparin component of the mixture, we find that, consistent with previous findings, crude heparin is less sulfated than purified heparin (Fig. 1) (2, 5). In addition to an overall increase in N-sulfo substituted disaccharides as well as 2-O-sulfidouronic acid and 3-O-sulfogalactosamine (accounting for the overall increase in specific activity), purified heparin has lower levels of undersulfated domains (15), including the linkage region (16), unsulfated glucuronic acid, and N-acetylgalactosamine residues (17). For example, the unsulfated glucuronic acid content in crude heparin (considering only the heparin/heparan sulfate component) is 40–50%, whereas in purified heparin the amount is closer to 18–22% (2, 5). Moreover, the N-acetyl glucosamine content in crude heparin is $\approx$30% whereas that of purified heparin is closer to 12–18% (17). Because of the intrinsic structural heterogeneity of HS and heparin, most of the NMR signals of HS overlap with those of heparin. However, HS

**Fig. 1.** HSQC analysis of crude heparin (black) compared with purified heparin (gold). Whereas crude heparin contains a number of components, purified heparin contains largely (or only) heparin. Signals associated with HA and C are identified in red; those arising from HA are shown in light blue. Signals from DS are shown in green, whereas those that arise primarily or exclusively from heparin are identified in black. $A_{\text{NS}}$, N-sulfogalactosamine; $A_{\text{NS}}$N-acetylglucosamine; L, iduronic acid; G, glucuronic acid; LR, linkage region.

Analysis of the tank bottom and the GAG waste material by PAGE and comparison to purified heparin indicates that the tank bottom material largely contains DS, CS C, and DS, with very little heparin present. Conversely, the GAG waste contains heparin and DS. PAGE has been shown to be able to resolve individual components of GAG mixtures (11). From this initial analysis, it is clear that undersulfated polysaccharide components are largely removed early in the heparin purification process, with DS being removed throughout the purification process. As such, the composition of side-stream byproducts changes as the purification process proceeds. We confirmed and extended PAGE analysis using $^1$H NMR (Fig. S4). In the case of tank bottom material, the proton NMR spectrum largely matches that of pure DS. In addition, the lack of appreciable signals at 5.2 and 5.5 ppm, assigned to the H-1 of 2-O-sulfido-L-iduronic acid and H-1 of N-sulfoglucosamine, respectively, indicates that tank bottom material likely contains mostly DS with very little heparin-like material. We confirmed this analysis through heteronuclear multiple quantum coherence (HMQC) analysis and comparison of the profile to a heparin reference standard (Fig. 2).
In contrast, analysis of the proton NMR spectrum for the GAG waste fraction indicates that it contains heparin and DS. Quantification of the signals for DS, including the N-acetylgalactosamine signal, indicates that GAG waste contains at least 10% DS. Other minor alterations in the proton NMR profile for the GAG waste compared with purified heparin are likely due to subtle alterations in the sequence composition of this material compared with purified heparin.

We also compared the tank bottom material to GAG waste material and to purified GAGs using capillary electrophoresis (Table 1, Fig. S5). Through this analysis, we find that the tank bottom material can tentatively be assigned as mostly containing DS. As expected from the above NMR-based analysis, GAG waste material appears to contain heparin, with detectable levels of DS. In addition, consistent with the presence of undersulfated sequences in the GAG waste, this material exhibited a wider range of sequence heterogeneity in CE analysis (exhibited as a higher polydispersity) compared with purified heparin, with a slightly changed migration time.

Therefore, taken together, these results indicate that CE and NMR can be important tools to assess the overall purity of heparin preparations. In addition, two-dimensional NMR analysis resolves individual components from one another, enabling increased confidence in both structural assignment, as well as ensuring the detection of different GAG components.

Analysis of Oversulfated Side-Stream Byproducts of the Heparin Purification Process. Having defined the composition of side-stream byproducts of the heparin purification process and their relationship to both crude and purified heparin, we next examined the ability of CE and NMR (both proton and two-dimensional NMR) to identify the presence of various persulfonated derivatives, both individually and in the context of a side-stream byproduct within heparin. Given our analysis of crude heparin as well as tank bottom and GAG waste side-stream material, we persulfonated CS, DS, and heparin individually as well as tank bottom and GAG waste material directly (Fig. S2). This analysis is of importance for several reasons: (1) based on findings in the literature, persulfonated polysaccharides, and not just OSCS, likely activate the contact system and thus, if present within a heparin preparation, might be capable of eliciting an adverse response; (2) persulfonated GAGs have been reported to have anticoagulant activity, primarily mediated through their pro-co-factor II (18) and thus their presence might not be detected by global anticoagulant assays; and (3) the presence of OSCS in heparin suggests that contamination with other persulfonated polysaccharides might be possible.

First, the tank bottom and GAG waste material were persulfonated (19), and analyzed by $^1$H (Fig. S6) and HMQC (Fig. 3) NMR to determine the chemical signatures associated with each. The spectra for these materials were also compared with OSCS-contaminated heparin and to isolated contaminant. Neither of the waste streams or their persulfonated products had spectral properties similar to the OSCS contaminant. Consistent with the analysis from above, persulfonated tank bottom material mostly resembled oversulfated DS. Oversulfated GAG waste was clearly distinguished from both OSCS-contaminated heparin and OSCS. Based on chemical shifts at $\approx$4.55 and 4.60 ppm (assigned to H-2 and H-3 of 3-O-sulfoiodouronic acid) and 4.40 ppm (assigned to H-3 of 3-O-sulfoiodouronic acid) (18), it appears that persulfonated GAG waste largely contains persulfonated heparin components. This assignment was confirmed through the use of CE. Analysis of a solution of heparin (+OSCS) spiked with either 10% wt/wt of persulfonated tank bottom or GAG waste material indicated that these materials are clearly distinguishable from both heparin and OSCS (Fig. 4). Finally, analysis of persulfonated crude heparin yields a complicated spectrum, as expected because it contains CS, DS, and HA as well as HS/heparin, which nonetheless yields signals, which can clearly be distinguished from either heparin, OSCS, or OSCS-contaminated heparin (Fig. S7).

To further this analysis, we persulfonated individual components of side-stream heparin, that is, CS, DS, and heparin, and determined whether these could be separated/identified as distinct from heparin. After persulfonation of CS, DS, and heparin, to create OSCS, OSDS, and OS heparin, respectively, we examined the proton NMR spectroscopy of the purified compounds (Fig. S8). Analysis of the region at $\approx$2 ppm, representing the N-acetyl portion of the spectra, indicates that, under conditions of identical sample concentration, pH, and temperature, the chemical shift for each persulfonated species, with the exception of oversulfated heparin (OS-heparin), was distinct from heparin’s N-acetyl signal. Analysis of the entire proton NMR spectra for these persulfonated GAGs indicate distinct differences for each compared with heparin. Consistent with persulfonation, OSCS, and OSDS have relatively simple proton spectra which can be readily defined and which are distinguished from that of heparin or OS-heparin. The spectrum for OS heparin is somewhat more complicated; however, there are differences in the one-dimensional (1D) $^1$H NMR spectrum for OS-heparin compared with that of heparin, including a pronounced

<table>
<thead>
<tr>
<th>Material</th>
<th>Migration time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td></td>
</tr>
<tr>
<td>Heparan Sulfate</td>
<td>9.8</td>
</tr>
<tr>
<td>Chondroitin Sulfate A</td>
<td>7.5</td>
</tr>
<tr>
<td>Chondroitin Sulfate C</td>
<td>7.0</td>
</tr>
<tr>
<td>Dermatan Sulfate</td>
<td>8.3</td>
</tr>
<tr>
<td>Heparin</td>
<td>5.0–5.1</td>
</tr>
<tr>
<td>Samples</td>
<td></td>
</tr>
<tr>
<td>Tank Bottom</td>
<td>8.3</td>
</tr>
<tr>
<td>GAG Waste</td>
<td>5.8, 8.3</td>
</tr>
</tbody>
</table>

![Fig. 2. HMQC of pure heparin (green), tank bottom (red), and DS (blue).](image-url)
signal from the H-2 and H-3 of 3-O-sulfo-N-sulfo glucosamine as well as the H-2 of 2-O-sulfo-3-O-sulfoiduronic acid (assignments are presented in Table S1). Taken together these results indicate that $^1$H NMR can be used as a tool to identify potential contaminants within heparin.

Given the fact that oversulfated heparin contains an N-acetyl region within the proton NMR spectrum that is similar to heparin’s, we also completed HSQC analysis of heparin mixed with 30% of OS heparin (Fig. 5). We find that oversulfated heparin can be detected in the HSQC spectrum. For example, consistent with the literature (18, 20, 21), we could readily detect signatures associated with 2-O-sulfo, 3-O-sulfo iduronic acid as well as signals associated with 3-O-sulfo-6-O-sulfo-glucosamine (Table S1). Based on the studies presented here, and others (17, 22), we estimate that a wide variety of contaminants, if present at a level >3–5%, will be detected in the HSQC under the experimental conditions used in this study; use of a higher field magnet with a cryoprobe can lower the limit of detection to <1%.

In parallel we also completed capillary electrophoretic analysis of OSDS and OS heparin alone and with OSCS-contaminated heparin.
Discussion

Since the initial report discovery and structural elucidation of OSCS in heparin (13), a number of analytical tools and methods have been used in the overall analysis and are described in a series of recent publications, including NMR (23–25), MS (26), IR-Raman (27, 28), CE (29–31), HPLC (32), biosensor (33), and bioassays (34–36). Also, the structural assignment of OSCS has been independently confirmed by other independent investigations (41), providing additional support to the fact that OSCS is the major contaminant within suspect heparin lots. In subsequent analysis of multiple lots of heparin, no other major contaminants have been detected; notably, DS impurities are seen in many preparations of pharmaceutical heparins. Thus, it appears that OSCS was added either inadvertently or purposely by itself, rather than in the context of a side-stream product. Nevertheless, we sought to define other potential contaminants and determine whether these contaminants, resulting from persulfonation of either a component or the entirety of side-stream heparin, could be detected and identified using a combination of NMR and CE.

One of the key definitional requirements for this study is to define what is meant by side-stream heparin or heparin byproducts. To this end, we analyzed crude heparin and compared the composition of it to the corresponding purified material. We find that crude heparin contains heparin and undersulfated polysaccharide components, including CS, DS, and HA. As such, crude heparin has a continuum of polysaccharide chains, both in terms of the type of polysaccharide, as well as their overall sulfation levels. Because of the intrinsic structural heterogeneity of heparin, most of the NMR signals of the lower sulfated heparin-like chains (referred to as HS) overlap with those of heparin, and HS impurities in heparin preparations can usually be detected through quantitative analysis, such as the relative increase of the intensity of signals associated with N-acetylgalactosamine- and glucuronic acid-containing sequences and the corresponding decrease of the relative intensity of signals associated with N-sulfogalactosamine and 2-O-sulfiduronic acid-rich sequences.

In the case of oversulfated heparin side-streams, we find that there are characteristic signatures which can be used to differentiate the presence of an oversulfated heparin side-stream with heparin. Consistent with the studies of Perlin and Yates, the presence of the unnatural 3-O-sulfiduronic acid (either with or without 2-O-sulfido group) is a key signature as is the presence of enriched levels of 3-O-sulfo, 6-O-sulfo, N-sulfogalactosamine residues (21, 37, 38). Moreover, if the iduronic-to-glucuronic ratio is maintained it can be determined whether persulfonation was completed on a heparin or on a HS- mixture. Finally, it is clear that persulfonation of heparin, and to a lesser extent HS, is complicated by the lability of N-sulfo groups. Thus, unless the product is subsequently N-sulfonated, another signature of OSHS/OS heparin is the presence of unsubstituted amino groups, which can be detected by NMR (typical signals at 3.36/57.1 ppm) (39) or by labeling of these moieties by an amine specific reagent (40). The need for a second N-sulfonation step to escape such detection would likely make contamination of heparin with oversulfated heparin (or oversulfated HS) uneconomical. Nevertheless, as mentioned above, persulfonated polysaccharides, including OS heparin/HS are expected to activate the complement system and have global anticoagulant activity, including activity mediated through heparin co-factor II activity.

From this study, we find that both NMR and CE are useful to distinguish purified heparin from heparin containing OSCS or other potential persulfonated derivatives of side-stream heparin or components of it. If completed in a carefully controlled manner with the appropriate reference material, the resolving power of these tests is significant and can, for example, distinguish OSCS, OSDS, and OS heparin from one another. One potential caveat is that analysis here involved persulfonated derivatives. Partially sulfonated derivatives have different chemical shifts in the NMR spectrum, different migration times in CE, and a more heterogeneous composition, complicating detection.

Finally, multidimensional NMR methods, such as HMQC and HSQC, as demonstrated here and elsewhere (13), are powerful techniques to resolve and identify potential contaminants in heparins. For example, in the case of OS-heparin spiking of heparin (Fig. 5), HSQC analysis clearly identifies signals associated with OS-heparin that are well resolved from those of heparin. Conversely, OS-heparin is much less distinguishable from heparin in the one-dimensional NMR experiments (Fig. S10). In conclusion, given the complexity of heparin samples, and the requirement to ensure their purity, it is clear that orthogonal analysis of heparin samples is critical. Of course, although CE and NMR are valuable analytical tools in this regard, other enzymatic approaches, including digestion, separation, and quantification of the resulting fragments, also provide important information. We demonstrate here how such orthogonal analytical strategies can detect a wide array of potential contaminants in heparin, including both OSCS, the major contaminant within suspect heparin lots, and other potential persulfonated polysaccharide components.

Materials and Methods

Pure heparin sodium salt (from porcine intestinal mucosa), heparan sulfate [(HS), from porcine intestinal mucosa], chondroitin sulfate A [(CS A), bovine trachea], and DS (from porcine intestinal mucosa) were purchased from Celsus. Contaminated heparin, isolated contaminant (OSCS), GAG waste, and tank bottom were provided by Baxter.

Persulfonation of Various GAGs, GAG Waste, and Tank Bottom. Fully sulfated GAGs were prepared according to a modification of a literature procedure (19) using sulfur trioxide-pyridine complex (PySO3) as the sulfonating agent. Briefly, the sodium salt of a GAG or GAG mixture (0.5 g) was converted to its tributylammonium (TrBA) salt. After freeze-drying, the GAG TrBA salt (0.7 g) was dissolved in anhydrous dimethylformamide (8 mL). Py SO3 (12 g) was then added and the reaction mixture was stirred overnight at 40 °C under an argon atmosphere. The reaction mixture was cooled to 0 °C and 16 mL of water was added to stop the reaction. Precipitation of the fully sulfated GAG waste was accomplished by the addition of 50 mL of absolute ethanol. Isolation afforded the GAG PyH+ salt (1.2 g) as a white powder. This material was dissolved in 10 mL water and a saturated solution of sodium acetate in ethanol (30 mL) was added. The resulting precipitate was recovered by centrifugation, washed with ethanol, and dried under vacuum, affording persulfonated GAG as the sodium salt (0.5–0.6 g).

NMR. Samples (10–20 mg) of pure heparin and mixtures of heparin containing GAG and/or contaminant were dissolved in 0.5 mL D2O (99.996%, Sigma or Euriso-Top) and freeze-dried 3 times to remove the exchangeable protons. The samples were redissolved in 0.5–0.6 mL D2O. Spectra were recorded at 300 K on Bruker Avance II 600 MHz or 800 MHz spectrometers equipped with a cryogenically cooled probe (HCN or TCI). The spectrum for Fig. 5 was recorded at 308 K on Bruker Avance II 600 MHz or 800 MHz spectrometers equipped with a 5-mm TCI probe.

Monodimensional 1H spectra were obtained with pretradation of residual HD2O signal, 32 scans, and a recycle delay of 12 s. Two-dimensional gradient enhanced HSQC spectra were recorded with carbon decoupling during acquisition with 320 increments of 16 scans for each. In all HSQC experiments the polarization transfer delay (D = 1/2 · T1C) was set with a 1JC–C coupling values of 150 Hz. The matrix size 1K × 512 was 0 filled to 4K by application of a squared cosine function before Fourier transformation. Chemical shift...
values were measured downfield from trimethylsilyl propionate sodium salt (TSP) in all spectra.

**CE.** Capillary electrophoresis was completed on an Agilent Technologies CE System using a modification of a published procedure (29). The buffer was 1 M Li Phosphate, pH 2.5. A 25-μm capillary was used with a 33.0-cm total length and a 24.5-cm effective length. The capillary temperature was kept constant at 20 °C. The voltage for the run was maintained at ~14 kV in reverse polarity. Injection of samples was completed by pressure injection at 500 mbar*s. All samples were detected at 195 nm. Individual components, either unmodified or persulfonated, were injected at a concentration of 5 mg/mL. In addition, for selected samples a 10% w/v solution of the persulfonated material was spiked into the USP System Suitability Reference Standard at 50 mg/mL.

**ACKNOWLEDGMENTS.** We thank Edward Chess and Edwin Moore (Baxter International, Round Lake, IL) for supply of materials and helpful discussions and Todd Wielgos, Mark Nordhaus, and Baxter International for technical assistance and expertise. This work was supported by National Institutes of Health Grants GM 57073 and HL59968 (to R.S.).