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Theme Issue Article

The Tainted Heparin Story: An Update

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Summary

Recently, certain batches of heparin have been associated with an acute, rapid onset of serious side-effects indicative of allergic-type reactions. These reports generated significant concern regarding the possible presence of a dangerous contaminant within heparin and highlighted the need to re-assess the purity criteria of heparin preparations for clinical use. Given the nature of the array of all possible contaminants, traditional screening tests cannot safely differentiate between contaminated and uncontaminated heparin preparations. Mono- and bi-dimensional NMR spectroscopy are powerful techniques that are able to detect and quantify a wide variety of potential sulfated polysaccharide contaminants. As such, these techniques are powerful tools for the analysis and assessment of heparin preparations.

Keywords

Magnetic resonance, heparins, glycosaminoglycans

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Introduction

In late 2007 and into early 2008, a spike in serious adverse events was associated with administration of heparin (1, 2). This led to a multifaceted investigation into the root cause for this dramatic increase in allergic-type reactions. Concomitant with the initial identification of the contaminant as "heparin-like" by the US FDA, screening methods were introduced, based on capillary electrophoresis (CE) and proton nuclear magnetic resonance (^{1}H NMR) (3). Subsequently, contaminated heparin lots were detected in a number of countries, including Germany, France, Italy, Japan, Denmark and Australia. Intensive efforts to analyse recalled heparin lots and determine the identity of the contaminant, including those of the authors, ultimately led to the identification of the contaminant as oversulfated chondroitin sulfate (OSCS). Key to this analysis was the use of multidimensional NMR, including HSQC (heteronuclear single quantum coherence) and HMBC (heteronuclear multiple bond correlation) experiments which determined the sulfation pattern of the disaccharide repeat, the epimeric state of the uronic acid, and the glycosidic linkage pattern which characterised the disaccharide repeat as a tetrasulfated chondroitin sulfate-like unit. Ultimate proof-of-structure was obtained when a synthesised version of OSCS matched the HSQC profile of the contaminant in heparin (Fig. 1) (4).

In concert with the analytical studies, biological characterisation of the contaminant correlated the presence and extent of contamination with anaphylactoid reactions. It was found that the contaminant was capable of activating the contact system both *in vitro* and *in vivo*, using pigs as a model system. In a follow-up study, epidemiological evidence indicated that patients exhibiting the allergic-like symptoms, including hypotension, nausea, and shortness of breath were found to likely have received OSCS-contaminated heparin (5).

Thus, taken together these studies enable some measure of resolution to the heparin crisis and point to the fact that OSCS was likely the causative agent in the onset of anaphylactoid responses in patients. Sufficient proof has been provided that the adverse effects observed for recalled heparins are correlated with their content of OSCS (5, 6). In addition, to our knowledge no additional serious anaphylactic events have been reported after the discovery of the OSCS contamination.

In response to these recent developments, including the development of screening tests for heparin, the United States Pharmacopoeia (USP) and European Pharmacopoeia (EP) requirements for heparin have been revised. The USP has indicated that

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the monograph for heparin, which currently includes the NMR and CE screening methods, will be further revised to incorporate additional testing. Similarly the EP monograph is also currently undergoing revision.

Framing the issue

These developments beg the question of what should the analytical testing strategy encompass? This question is especially pertinent in the context that OSCS may have been deliberately

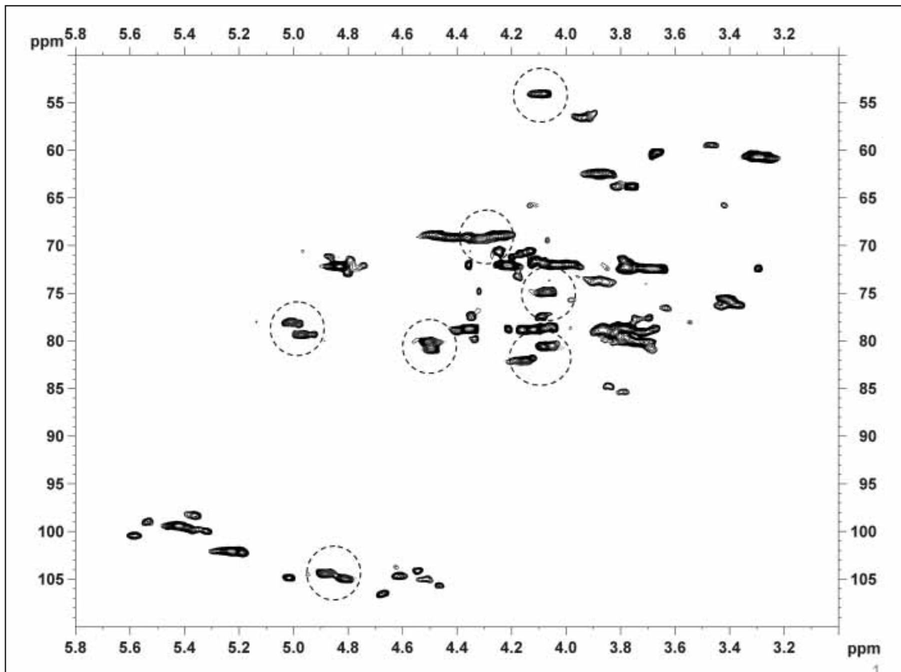

Figure 1: HSQC spectrum of the contaminated sample (circled signals) overlaid on control sample (4).

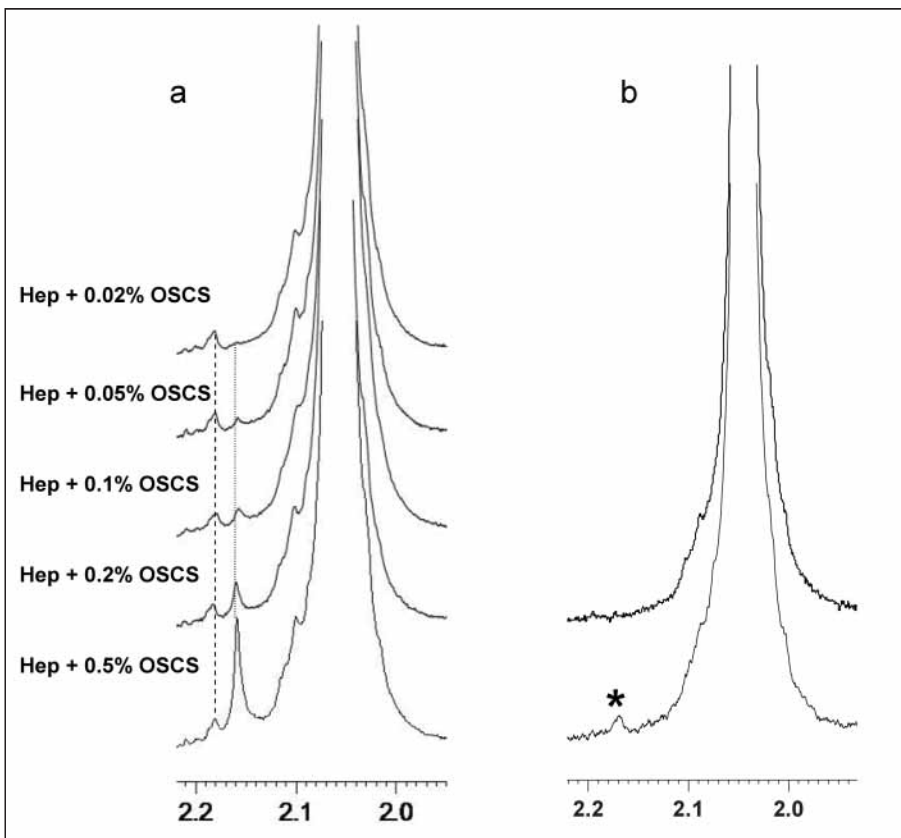

Figure 2: N-acetyl region of the proton spectra of heparin in the absence and in the presence of OSCS. A) N-acetyl region of the 500 MHz proton spectra recorded at 25°C of heparin spiked with different amount of OSCS. ^{13}C satellite bands of the heparin N-acetyl peak (dashed line) and signals of the OSCS N-acetyl peak (dotted line), are shown. A limit of detection of 0.05% (w%) was determined. B) N-acetyl region of the proton spectrum of heparin before (below) and after ^{13}C decoupling during acquisition at the frequency of the N-acetyl carbon signal (24 ppm) with a power of 0.26 Watt. The non-decoupled spectrum shows the N-acetyl satellite signal (*). Samples were prepared dissolving 25 mg of OSCS/heparin mixtures in 0.6 ml of deuterium oxide obtaining a solution pH of 6.8.

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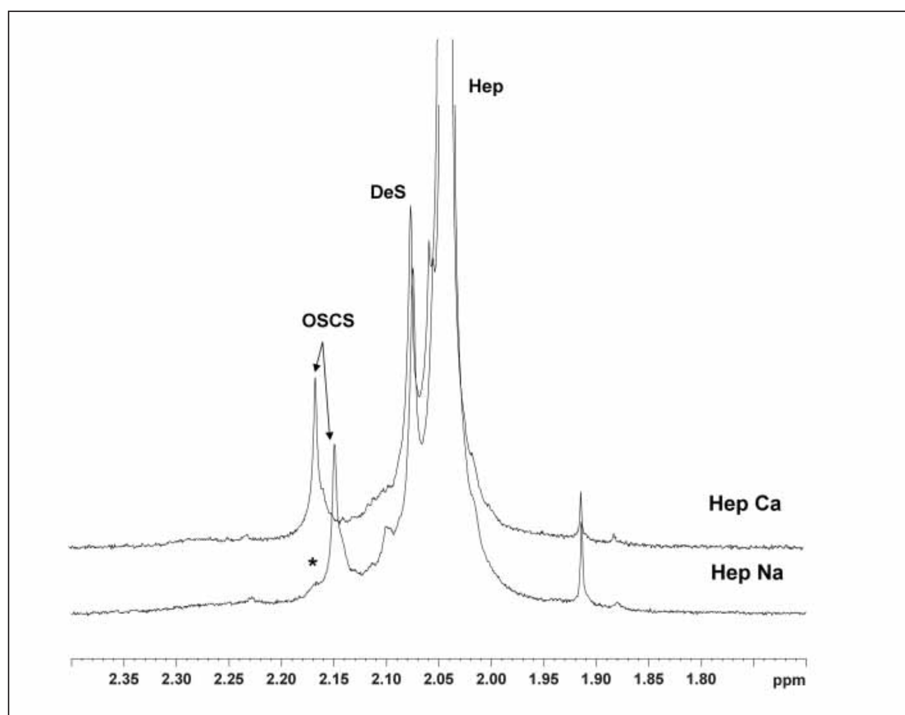

Figure 3: N-acetyl region of the 500 MHz proton spectra recorded at 25°C of sodium (Hep Na) and calcium (Hep Ca) heparins containing 3% of dermatan sulfate (DeS) and 2% of OSCS. Change in counter ion causes a 17 Hz downfield shift of the OSCS N-acetyl peak and less than 1–2 Hz shift of the dermatan sulfate and heparin N-acetyl peaks. Samples were prepared dissolving 25 mg of OSCS/heparin mixtures in 0.6 ml of deuterium oxide obtaining a solution pH of 6.8.

added to heparin preparations. Thus, any analytical test, or series of tests, should be able to assess the purity of heparin and be able to detect the presence of a wide variety of contaminants, if present. To develop such a paradigm, one of most fundamental activities is to define *heparin sodium* as a complex pharmaceutical agent made of a mixture of polysaccharide chains.

The majority of heparin contains a disaccharide repeat unit of uronic acid 1→4 linked to N-acetyl or N-sulfo glucosamine. Each disaccharide unit can be differentially sulfated; however the extent of sulfation for pharmaceutical grade heparin is fairly uniform due to the biological sourcing of heparin and the steps employed in its purification (7).Therefore, a reasonable analytical testing strategy should measure these aspects of the mixture, ensuring the appropriate disaccharide unit composition and the "correct" sulfation pattern. However, this is complicated by the fact that heparin is extracted from porcine intestinal mucosa and purified through a combination of chemical treatment, differential precipitation and capture by anion exchange resin. As part of this purification process, other natural glycosaminoglycans accompany heparin and, additionally some parts of heparin are lost and/or modified. For example, dermatan sulfate often co-purifies with heparin and, in fact, its industrial-scale removal from heparin implies selective loss of heparin species – *(*Liverani et al., *this Theme Issue)*- an undesirable outcome. As such, some amount of dermatan sulfate is tolerated in heparin, and, generally, a <1% dermatan sulfate content in heparin is considered safe (8).

Heparin preparations have historically been analysed by proton NMR, and this technique forms the basis for one of the FDA screening tests. Previous studies have identified the fact that proton NMR can be used to assess compositional features of heparin as well as detect the presence of impurities such as dermatan sulfate (9). Our analysis of over 50 heparin preparations

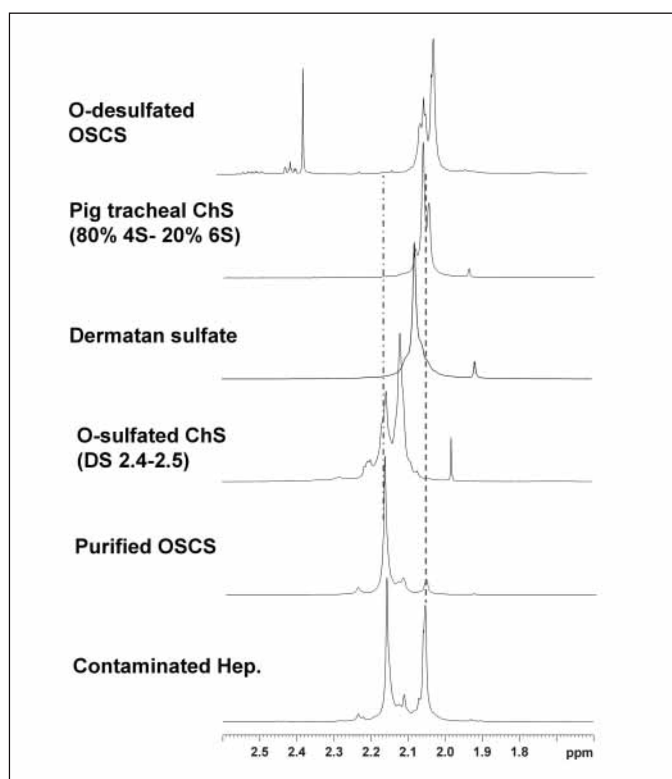

Figure 4: N-acetyl region of 500 MHz proton spectra of different natural and chemical modified glycosaminoglycans in comparison with purified OSCS, contaminated heparin and extensively O-desulfated OSCS. ChS, chondroitin sulfate; DS, sulfation degree.

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by this method has highlighted its considerable strengths as well as its weaknesses. It has proven to be able to specifically detect the presence of OSCS in heparin down to a level of 0.1% or better, depending on the field strength of the instrument. In addition, the proton NMR pattern is information rich and can be used to assess aggregate properties of the polysaccharide chains, including sulfation pattern (9). However, it must be recognised that this methodology suffers from a number of shortcomings which limit its usefulness in assessing overall quality attributes of various heparin preparations, especially if one focuses only on the N-acetyl region of the spectrum (2.0–2.2 ppm). For example, ^{13}C satellite bands of the heparin N-acetyl peak complicate assignment of OSCS (Fig. 2). As mentioned above, the limit of detection of OSCS is dependent on magnetic field strength and is also

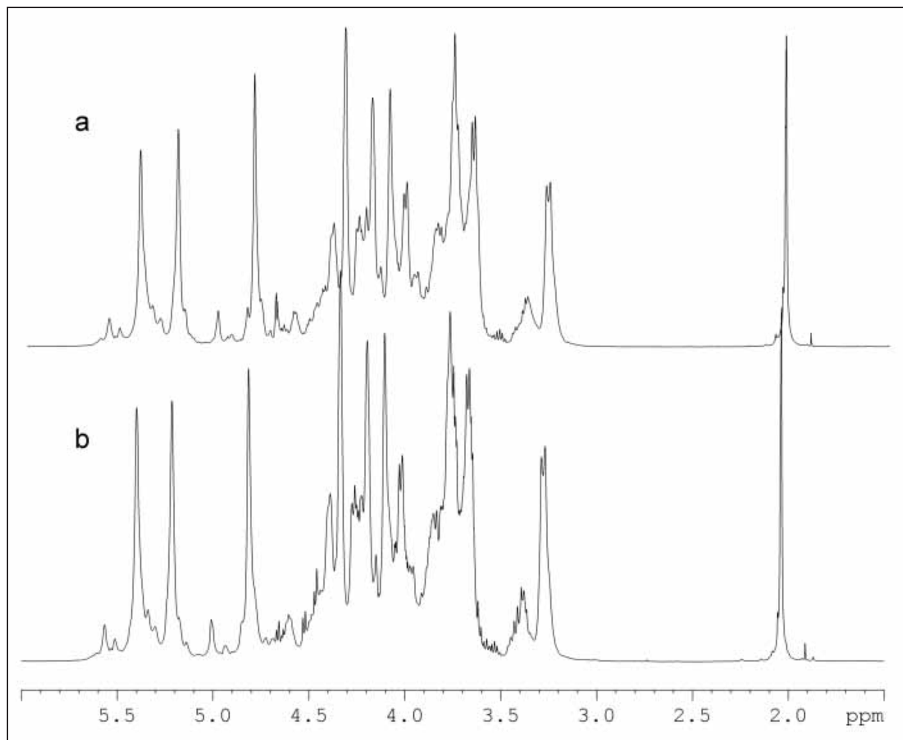

Figure 5: 500 MHz proton spectra of heparin (A) and heparin spiked with 4% of alginate sulfate (B).

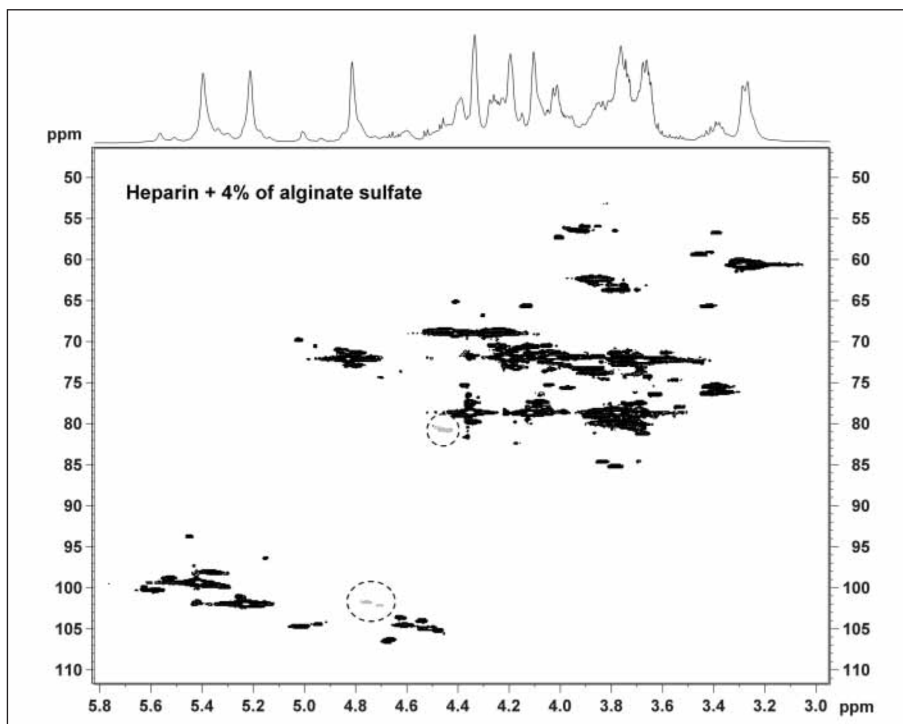

Figure 6: 500 MHz HSQC spectrum of heparin spiked with 4% of alginate sulfate. Signals of alginate sulfate that do not overlap those of heparin are circled in gray. The HSQC spectrum was recorded at 35°C with carbon decoupling during acquisition with 320 increments of 32 scans for each. Polarisation transfer delay ($\Delta = 1/[2\ ^1J_{C-H}]$) was set with a $^1J_{C-H}$ coupling values of 150 Hz. The matrix size 1K 512 was zero filled to 4K 2K by application of a squared cosine function prior to Fourier transformation. Chemical shift values were measured downfield from trimethylsilyl propionate sodium salt (TSP).

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dependent on the content of dermatan sulfate in the sample. For instance, the presence of 15%-20% of dermatan sulfate in drugs constituted by glycosaminoglycan mixtures reduces the limit of detection of OSCS at 500MHz from 0.05% to 0.2%. Finally, peaks can shift based on the counterion present, *i.e.* calcium versus sodium (Fig. 3), as well as the exact sulfation pattern of the heparin and/or the contaminant.(10) In fact, through subtle variation of the sulfation pattern of OSCS, the N-acetyl peak can largely occur at the same chemical shift as that of heparin, confusing the interpretation (Fig 4). Finally, this method was designed to specifically screen for the presence of OSCS. Detection and quantification of NMR N-acetyl peaks would not reveal non-N-acetylated potential contaminants such as polysulfated alginate and N-sulfated heparinoids. For example, polysulfated alginate is not detected in the proton NMR spectrum at a level of four percent (Fig. 5).

Based on our own experience with the analysis of heparin, we find that two dimensional NMR analysis is a more powerful technique that can be used to reveal most, if not all, non-heparin, polysaccharide-based species potentially present in heparin preparations (11). Multidimensional NMR analysis of the same heparin sample from above, spiked with polysulfated alginate, (Fig. 6) reveals clearly identifiable signals associated with the alginate backbone and, thus, this sample can be distinguished from a clean heparin preparation. Based on a series of analyses, we find that, by considering the entire 2D spectrum, we can routinely differentiate clean heparin from spiked heparin for a wide variety of potential contaminants when the levels of the potential contaminant are greater than 2–4% (weight percent for polymeric compounds) Actually, application of the method to heparin/heparan sulfate preparations indicated that at 500MHz signals corresponding to components present in amounts higher than 2–4% can be accurately quantified (11).

Thus, taken together, these results strongly point to the fact that 2D NMR can be a powerful tool in the analysis and assessment of heparin.

Conclusions and proposals

With a complex mixture such as heparin, to ensure the purity and relative uniformity of the material requires the use of multiple, orthogonal analytical techniques. In addition, we must recognise that certain analytical techniques yield greater information on the mixture than do others. For example, we demonstrate here that two dimensional NMR is able to detect and quantify a wide variety of potential polysaccharide contaminants, such as polysulfated alginate, which are not detected by a simpler proton NMR methodology. Indeed, multidimensional NMR was the key analytical tool which enabled the structural elucidation of OSCS in heparin (4).

This example also highlights the fact that, in the analysis of a complex mixture such as heparin, the specificity of a method must be rigorously tested and supported by complementary testing. In addition, while it is clear that multidimensional NMR is an excellent tool to characterise heparin at a molecular level as well as to identify the presence of non-heparin material within the mixture, other strategies that yield the same information content can be devised. Any strategy which probes heparin's characteristics at this molecular level, such as potentially compositional analysis after enzymatic or chemical digestion, if properly implemented and potentially supplemented with additional testing, can control heparin to the same extent. Also, it must be recognised that additional testing should be completed on heparin, especially impurity testing, including testing for levels of nucleic acid, and protein.

In summary, we have demonstrated here three separate, but related, points. First, while the existing FDA tests provide an excellent screen for OSCS (defined as ~4 sulfates per disaccharide), additional controls are necessary to not only ensure the absence of OSCS but also to ensure the purity of a given heparin preparation. Second, we demonstrate that multidimensional NMR can resolve contaminants which are not observed by proton NMR. This fact highlights the significant resolving power of multidimensional NMR and its value as a method to interrogate various heparin preparations. Finally, for complex mixtures, we suggest assessing the molecular properties of the mixture through the use of orthogonal analytical techniques is important for appropriate understanding of heparin and, accordingly, its control.

The heparin contamination crisis has highlighted the importance of designing and implementing appropriate analytical tests as controls, tests which are able to measure molecular properties of the complex mixture. Development of such a testing scheme and its adoption by manufacturers of heparin is critical to ensure the integrity of the heparin supply chain and avoid a potential repeat of the heparin crisis.

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