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Review

Electrophoresis for the analysis of heparin purity and quality

The adulteration of raw heparin with oversulfated chondroitin sulfate (OSCS) in 2007–2008 produced a global crisis resulting in extensive revisions to the pharmacopeia monographs and prompting the FDA to recommend the development of additional methods for the analysis of heparin purity. As a consequence, a wide variety of innovative analytical approaches have been developed for the quality assurance and purity of unfractionated and low-molecular-weight heparins. This review discusses recent developments in electrophoresis techniques available for the sensitive separation, detection, and partial structural characterization of heparin contaminants. In particular, this review summarizes recent publications on heparin quality and related impurity analysis using electrophoretic separations such as capillary electrophoresis (CE) of intact polysaccharides and hexosamines derived from their acidic hydrolysis, and polyacrylamide gel electrophoresis (PAGE) for the separation of heparin samples without and in the presence of its relatively specific depolymerization process with nitrous acid treatment.

Keywords:
Capillary electrophoresis / Heparin / Oversulfated chondroitin sulfate / PAGE
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1 Introduction

Heparin is a linear sulfated natural polysaccharide consisting of 1→4 linked pyranosyl uronic acid (uronic acid, either α-L-iduronic, and β-D-glucuronic acid, with some *O*-sulfo substitution) and 2-amino-2-deoxyglucopyranose (α-D-glucosamine with either *N*-sulfo or *N*-acetyl substitution) repeating units (Fig. 1). It belongs to the family of glycosaminoglycans (GAGs) endowed with anticoagulant and antithrombotic properties used clinically over the last half-century as an anticoagulant drug [1–3].

Like all other GAGs, heparin is a polydisperse mixture containing a large number of chains having different molecular mass [4, 5]. The chains making up polydisperse pharmaceutical-grade heparin range from 5000 to over 40000 Da and contain a significant level of sequence heterogeneity [5]. In fact, depending on the origin, heparin is composed of a major (75–95%) trisulfated disaccharide repeating unit (Fig. 1), as well as a number of additional minor disaccharides structures corresponding to its variable sequences (Fig. 1).

Heparin and other GAGs are generally extracted and purified from animal tissues, in particular from bovine and porcine, and few publications or patents describe commonly used pharmaceutical processes [5]. The purification of the raw heparin is performed under cGMP conditions and it is designed to deal with potential impurities originating from the starting material or introduced during heparin extraction. Such impurities may be in the form of other GAGs, extraneous cationic counter ions, heavy metals, residual proteins or nucleotides, solvent, salts, bacterial endotoxins, bioburden, and viruses [5].

Unfractionated native heparin possesses several undesirable side effects that include dangerous hemorrhagic complications [6]. It was for this reason that low-molecular-weight (LMW)-heparins (average molecular mass of 3000–8000) were introduced as heparin substitutes having reduced side effects, more predictable pharmacological action, sustained antithrombotic activity, and improved bioavailability [7, 8]. While both heparin and LMW-heparins are parenteral agents, only LMW heparins show significant subcutaneous bioavailability.

In 2007–2008, patients presented, within several minutes after intravenous infusion of heparin, angioedema, hypotension, swelling of the larynx and related symptoms, which in some cases ended in death [9]. A synthetic oversulfated chondroitin sulfate (OSCS) (Fig. 1) [10, 11], not found in nature, was identified as a fraudulent contaminant in heparins

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Abbreviations: **ATR-IR**, attenuated total reflectance-infrared; **CS**, chondroitin sulfate; **DS**, dermatan sulfate; **FDA**, Food and Drug Administration; **GAG(s)**, glycosaminoglycan(s); **LMW**, low molecular weight; **OSCS**, oversulfated chondroitin sulphate; **PT**, prothrombin time; **USP**, United States Pharmacopoeia

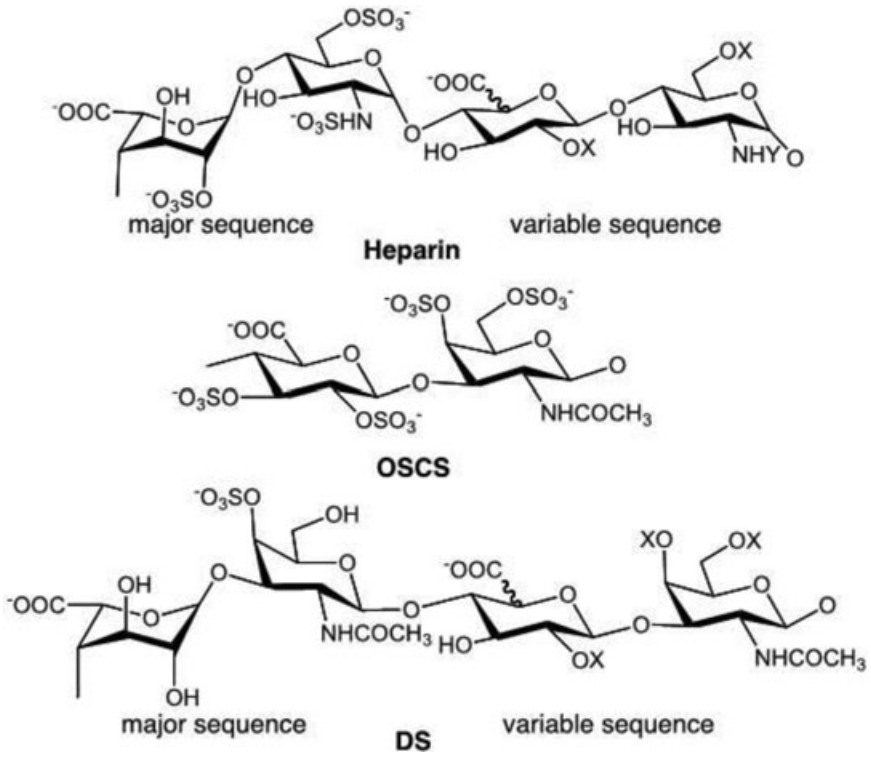

Figure 1. The structures of the major and minor repeating disaccharides comprising heparin (where X = SO_3^- or H, and Y = SO_3^- or $COCH_3$), oversulfated chondroitin sulfate (OSCS), and of the major and minor repeating disaccharides of dermatan sulfate (DS) (where X = SO_3^- or H). The structure of the OSCS contaminant, present within specific lots of heparin, has been fully identified by using multiple orthogonal techniques, including multidimensional NMR, to overcome the challenges inherent in the analysis of complex polysaccharides, including heparin [10]. The structure of OSCS was definitively confirmed as formed of disaccharide repeat units of D-glucuronic acid linked β1→3 to a β-*N*-acetyl-D-galactosamine (GalN). The disaccharide unit was found to posses an unusual sulfation pattern being sulfated at the 2 and 3 positions of the glucuronic acid as well as at the 4 and 6 positions of the GalN unit [10].

originally sourced from China and distributed in Europe and USA. Furthermore, this OSCS or its LMW derivatives were also present in LMW heparins. This OSCS contaminant is believed to have been added to certain batches of raw heparin in China in a deliberate act of adulteration. The degree of difficulty to detect this adulterant in an LMW heparin depends on the depolymerization process used in its manufacture [11]. After disclosure of these findings, the presence of significant amounts (1–10%) of dermatan sulfate (DS) was also observed as an impurity in heparin. However, due to the natural occurrence of this natural polysaccharide in tissues, from which heparin is extracted, the United States Food and Drug Administration (FDA) defined this as "process-related impurities" in heparin preparations. The United States Pharmacopoeia (USP) recommended limiting DS/CS impurities to not more than 1% [12]. Moreover, the Committee for Medicinal Products for Human Use of the European Medicines Agency was of the opinion that LMW heparins containing less than 5% of OSCS could continue to be used, avoiding intravenous route, until they are replaced by OSCS-free batches [13]. As a consequence, specific and sensitive analytical techniques are required for the detection of very low percentages of several possible contaminants different for structure and physicochemical properties. In fact, due to the nature of OSCS, traditional screening tests and analytical approaches are unable to differentiate between contaminated and uncontaminated lots, and several contaminated lots of heparin had passed the standard compendial tests. Consequently, the USP and European Pharmacopeia revised their monographs for heparin sodium. The USP examined a number of methods for inclusion in its revised monograph including capillary electrophoresis (CE), NMR methods [14,15], and anion exchange-HPLC.

NMR methods for determining heparin purity and presence of OSCS have been evaluated by a number of laboratories [16–23]. The integral or peak intensity of *N*-acetyl signal in optimized ^{1}H NMR is sufficient to quantify and limit DS impurity and OSCS contaminant in heparin using quantitative NMR spectroscopy [21–23]. The chemical shift of OSCS *N*-acetyl methyl signal, however, depends on counter-ion type and concentration. The chemical shift of the methyl signal of pure OSCS varies linearly from 2.13 ppm to 2.18 ppm with increasing amounts of Ca^{2+}, until reaching the saturation point at four Ca^{2+} ions per tetrasulfonated disaccharide unit [16,17]. By this analytical approach, OSCS was found in heparin samples in amounts ranging from 0.5% to 28%, mostly between 5% and 15% [22]. Furthermore, ethanol was found in varying amounts up to about 9.5% along with traces of acetone, formic acid, sodium acetate, and methanol [22].

Weak anion-exchange HPLC has also been used for the identification and separation of the intact heparin and OSCS and has been coupled to on-line UV (at 215 nm) and on-flow ^{1}H NMR detection [19]. Intact GAGs can be separated using a salt gradient from 0.1 M to 1.0 M NaCl at a solution pH of 10.25. However, multidimensional NMR [10], was judged to be too complex and expensive to be considered and it is viewed as not generally useful for quality assurance in quality control laboratories due to its incapacity to process many samples in a short time. Infrared spectroscopy has also been suggested as a potential method for the evaluation of heparin purity and

for detection of OSCS [24]. In fact, it can afford automatic data analysis by applying multivariate data analysis to the attenuated total reflectance-infrared (ATR-IR) spectroscopy.

2 Capillary electrophoresis

CE has emerged as a promising separation technique for non-sulfated and sulfated intact GAGs and GAG-derived oligosaccharides and disaccharides [25]. While not included in the revised pharmacopeial monographs because of insufficient time for assay validation, electrophoresis is capable of affording concentration and structural characterization data in quality assurance/quality control laboratories due to the high resolving power, sensitivity, and throughput of these techniques [25, 26]. The CE separation mechanism is based on molecular charge-to-size ratio, which is particularly useful for the analysis of polyanions such as heparin and OSCS. However, due to its heterogeneous nature, typically a relatively wide peak is observed for intact heparin in CE [27, 28], which complicates the separation from structurally related contaminants. The initial CE separation useful to detect OSCS in heparin preparations was performed by using phosphate buffer-based reversed polarity methods [28, 29]. However, only partial separation of OSCS and heparin could be achieved with the contaminant appeared as a leading edge peak only partially resolved from the broad peak associated with heparin. Furthermore, the limited resolution hindered the reliable determination of levels of OSCS and DS in heparin samples. A following CE method [30] used a high molarity sodium phosphate buffer to improve resolution, but no full separation of OSCS and heparin was still achieved with this background electrolyte.

In a further recent study, Somsen et al. [31] were able to enhance the OSCS-heparin separation by CE without compromising the simplicity of the method, in particular by using standard equipment with UV absorbance detection and requiring no sample pretreatment. By using high concentration of Tris-phosphate buffer at pH 3.0 as background electrolyte (BGE), excellent separation of OSCS and DS from heparin was achieved within 17 min (Fig. 2). A crucial parameter to allow high molarity buffers and obtain full separation is the replacement of sodium ions by Tris, as these ions contribute less to BGE conductance but still provide the same ionic strength. High currents and excessive Joule heating were prevented by employing fused-silica capillaries with an internal diameter of 25 μm. The method permitted OSCS and DS determination in heparin down to the 0.05% and 0.5% (w/w) level, respectively. CE application to contaminated heparin samples and different heparin standards also demonstrated the utility of the method to allow determination of the common DS impurity.

In another study [32], a mixed polymeric electrokinetic chromatography (EKC) system has been developed for the simultaneous determination of OSCS and DS in heparin samples. The EKC system consisted of 0.5% w/v polymeric β-cyclodextrin (β-CD), 0.4% w/v poloxamine copolymer Tetronic® 1107 and 400 mM Tris-phosphate buffer at pH 3.5. The highly sensitive method developed (Fig. 3) showed low values of LOD, 0.07% for OSCS, and 0.1% for DS, and values of LOQ of 0.2% for OSCS and of 0.3% w/w for DS with a concentration level of heparin sample as low as 0.1 mg/mL. Owing to its simplicity, high sensitivity, and reliability, the proposed method can be an advantageous alternative to the traditional methodologies for the analysis of heparin in raw material, especially in finished products because of the low amounts of heparin sample required.

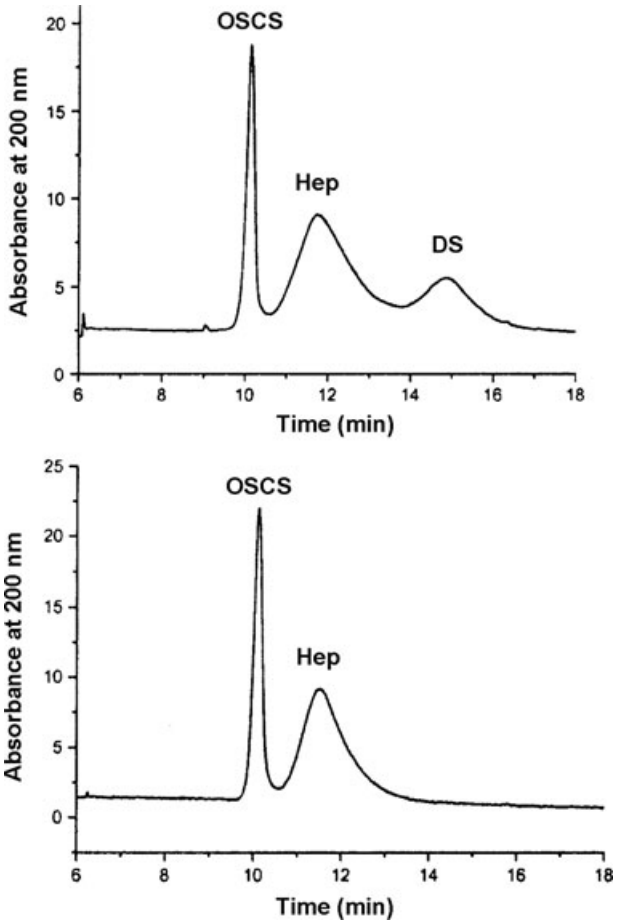

Figure 2. CE-UV of OSCS-contaminated heparin samples (modified from [31]). Sample concentration: 50 mg/mL. Capillary id: 25 μm. Total capillary length: 60 cm. Buffer: 850 mM Tris-phosphate pH 3.0. Capillary temperature: 35°C. Sample injection: 48 s at 2.0 psi. Voltage: −30 kV. Voltage ramping time: 5.0 min.

A different innovative CE approach has been developed and applied by Volpi et al. [33]. After degradation of the polysaccharides by acidic hydrolysis, the different hexosamines produced were derivatized with anthranilic acid and separated by means of CE in approximately 10 min (Fig. 4) with high sensitivity detection at 214 nm. Furthermore, anthranilic acid-derivatized hexosamines showed quite similar molar absorptivity, allowing direct and simple quantification of OSCS in heparin samples. Moreover, a preliminary step of specific enzymatic treatment by using chondroitinase ABC

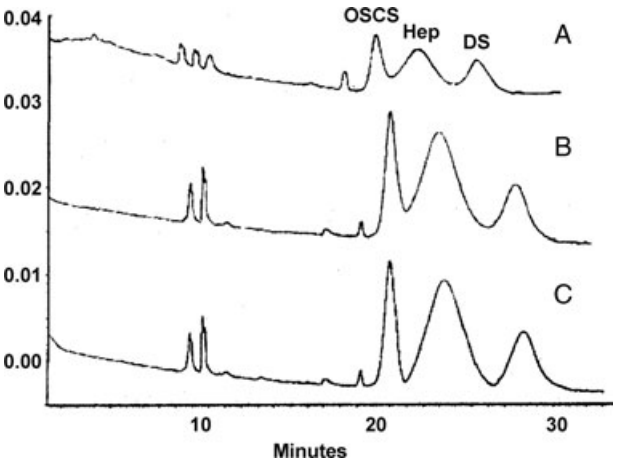

Figure 3. Electropherograms of OSCS, heparin, and DS under different background electrolyte. (A) 400 mM Tris-phosphate pH 3.5, (B) with 0.5% polymeric-β-CD, and (C) with 0.5% polymeric-β-CD and 0.4% Tetronic® 1107. Modified from [32].

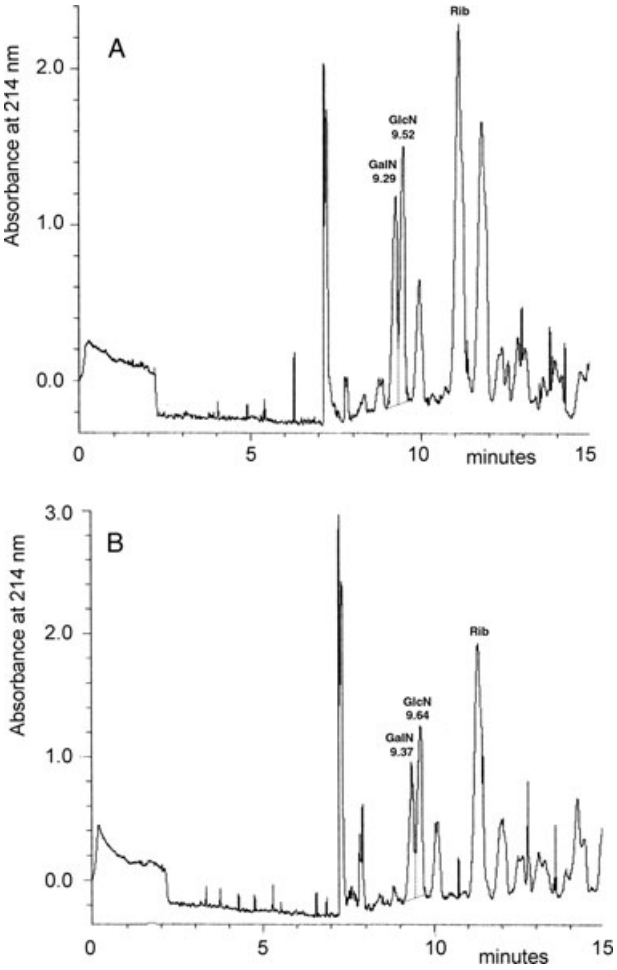

Figure 4. CE electropherograms at 214 nm of glucosamine (GlcN) and galactosamine (GalN) derivatized with anthranilic acid and obtained after hydrolysis procedure of (A) a contaminated raw heparin or (B) a formulated contaminated heparin sample. Ribose (Rib) is used as an Internal Standard. Modified with permission from [33].

may be applied for the specific elimination of interference in the analysis due to the possible presence in heparin samples of natural CS/DS impurities, making this analytical approach highly specific for OSCS. As little as 1% OSCS contaminant in heparin sample could be detected and quantified. While fluorescent labeling requires additional analytical steps, it offers certain advantages over the direct detection of OSCS, including increased sensitivity and improved resolution.

3 Polyacrylamide gel electrophoresis (PAGE)

PAGE provides a versatile method for the separation and partial physicochemical characterization of molecules by exploiting differences in size, conformation, and charge [34]. As a complementary screening technique to NMR, PAGE was applied in a comprehensive analysis of 31 heparin samples prepared from 1941 through 2008 [35]. OSCS in heparin samples was detected by PAGE after nitrous acid treatment, a relatively specific depolymerization process for heparin due to the presence of nitrous acid-sensitive *N*-sulfo groups that are absent on OSCS and most heparinoids containing hexosamine residues substituted by *N*-acetyl groups. Using this approach followed by PAGE analysis, OSCS was quantified in different proportions in mixtures with heparins. Semi-quantitative gel analysis was also performed and the standard curves showed excellent linearity with LOD values for heparin impurities ranging from 0.1% to 5%. Nitrous acid was also able to detect 17 heparinoids (Fig. 5). Furthermore, heparan sulfate and chitosan sulfate, having *N*-sulfo groups, were partially depolymerized by nitrous acid treatment. The remaining heparinoids containing only *N*-acetyl groups or have no amino sugars were stable to nitrous acid treatment and easily detected by PAGE analysis.

PAGE was also used to characterize the sensitivity of OSCS to different depolymerization processes used in LMW-heparin production [11]. On the basis of this analysis, OSCS was found to be sensitive to base-catalyzed β-eliminative cleavage and H_2O_2 treatment (Fig. 6). Oxidative depolymerization by H_2O_2 converted OSCS to monosaccharides at pH = 2 while longer oligosaccharides were observed at pH = 5 and pH = 7. OSCS was resistant to heparinase I treatment, and due to its structural features, both nitrous acid (due to the lack of a nitrous acid sensitive *N*-sulfo group) and periodate (due to lack of a vicinal diol moiety) failed to degrade OSCS (Fig. 6). As a consequence, PAGE analysis of OSCS contaminated LMW heparin samples successfully detected it among other possible impurities following nitrous acid treatment.

4 Other analytical methods

Heparin samples have also been tested with biochemical assays, such as the coagulation assay prothrombin time (PT) and in a two-step assay [36–38] consisting of incubation with

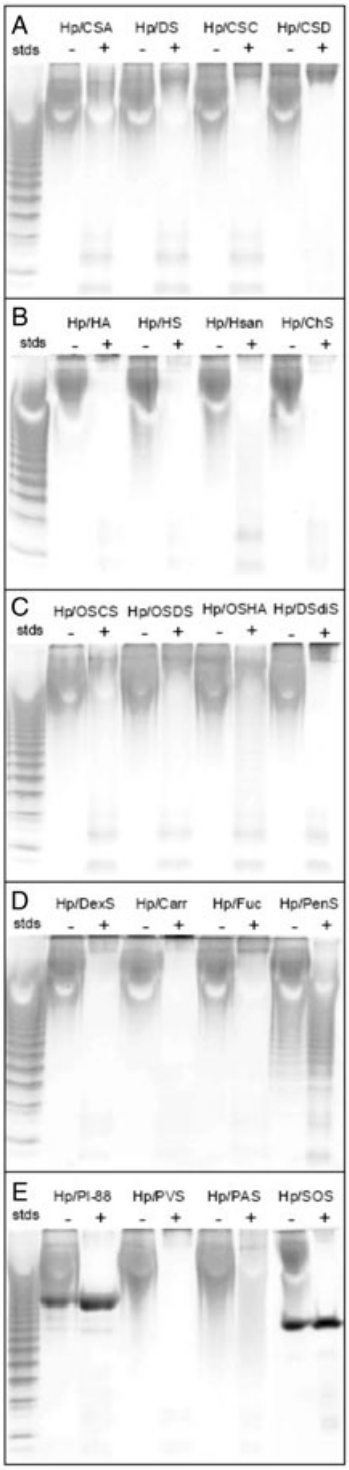

Figure 5. PAGE analysis of mixture of heparinoids and heparin after nitrous acid treatment. The first lane in each gel (A–E) contains mixture of heparin oligosaccharide standards. The remaining lanes in each gel contain mixtures of heparin and heparinoid (25%) untreated (−) and nitrous acid treated (+). Hsan, heparosan; Chs, chitosan sulfate; Dexs, dextran sulfate; Carr, carrageenan; Fuc, fucoidan; PenS, pentosan sulfate; PVS, poly(vinyl) sulfate; PAS, polyanethol sulfonate; and Hp, heparin. Reprinted with permission from [35].

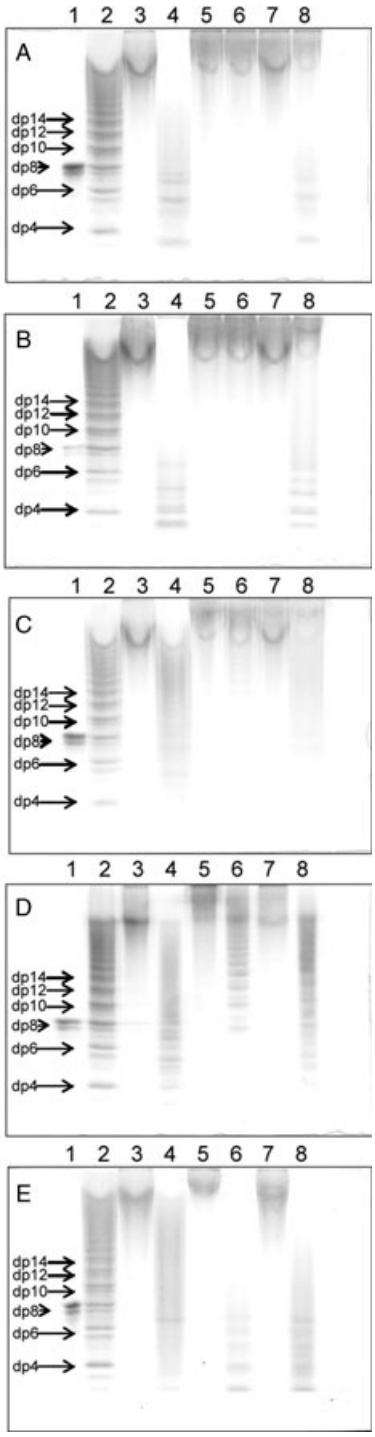

Figure 6. PAGE analysis of heparin, OSCS, LMW-heparins, and degraded OSCS. (A) PAGE analysis of samples from nitrous acid degradation. (B) PAGE analysis of samples from enzymatic degradation. (C) PAGE analysis of samples from periodate oxidation. (D) PAGE analysis of samples from alkaline treatment. (E) PAGE analysis of samples from hydrogen peroxide degradation, in which 1–8 are: 1, octasaccharide, derived from heparin. 2, heparin oligosaccharide standards. 3, heparin. 4, depolymerized heparin. 5, OSCS. 6, depolymerized OSCS. 7, 1:1 mixture of heparin/OSCS. 8, depolymerized 1:1 mixture of heparin/OSCS. Reprinted with permission from [11].

heparinase I followed by either a fluorescence measurement or chromogenic factor Xa assay. OSCS shortens the PT at plasma concentrations less than or equal to 10 μg/mL with the most pronounced effect at 1 μg/mL, whereas heparin as well as DS and CS do not modify the coagulation time. Another method able to quantify sulfated polysaccharides is the Inc-PolyH assay that relies on a fluorescent sensor molecule Polymer-H [39]. OSCS and heparin increase the fluorescence intensity of polymer-H linearly in a concentration dependent manner, and the content of OSCS in contaminated heparin can then be determined after removal of heparin.

5 Conclusion

Heparin is one of the oldest drugs currently still in widespread clinical use. It is unique because it was among the first biopolymeric drugs, and it is one of only a few carbohydrate drugs. However, this macromolecule is difficult to monitor and to analyze due to its polydisperse and heterogeneous nature. Therefore, alternative methods of controlling and regulating the production of heparin are urgently needed, and such procedures might represent the best long-term solution.

The FDA continues to develop and apply new methods with better sensitivity and selectivity to the analysis of complex biologically derived pharmaceuticals like heparin. For example, NMR experiments were central in the identification of the contaminant as OSCS [10]. However, generally, the more sensitive methods require expert operators and more sophisticated instrumentation (e.g. high-field NMR or LC-MS) with a concomitant added cost to the analysis. As a consequence, electrophoretic techniques may be of great importance to assist more complex analytical approaches for the determination of quality and purity of heparin and related quantitation of possible contaminants due to their high resolving power, efficiency, and sensitivity along with their generally high-throughput nature.

The authors have declared no conflict of interest.

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