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Quantitative compositional analysis of heparin using exhaustive heparinase digestion and strong anion exchange chromatography

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ABSTRACT

Heparin is a linear sulfated polysaccharide widely used therapeutically as an anticoagulant. It is also the starting material for manufacturing low-molecular-weight heparins (LMWH). Quality control of heparin and LMWH is critical to ensure the safety and therapeutic activity of the final product. However due to their complex and heterogeneous structure, orthogonal analytical techniques are needed to characterize the building blocks of heparin. One of the state-of-the-art methods for heparin analysis is based on complete enzymatic digestion using a mixture of heparinases I, II, and III, followed by the separation of the resulting oligosaccharides by liquid chromatography. The European Pharmacopoeia strong anion-exchange chromatographic method, used to quantify 1,6-anhydro derivatives in enoxaparin, is here applied to the analysis of the heparin building blocks. Their quantification, namely the determination of their average w/w percentage in the heparin chain, is obtained after identification of all components including glycoserine derivatives and 3-O sulfated di- and tetrasaccharides. This work therefore provides a comprehensive overview of the building blocks of unfractionated heparin, including those chemically modified by the manufacturing process, either within the polysaccharide chain or at its reducing end.

1. Introduction

Heparin is a highly sulfated polymeric linear chain with repeating disaccharide units comprised of an uronic acid (glucuronic or iduronic) and a glucosamine. Heparin is widely used as an anticoagulant in the treatment of thromboembolic diseases either as unfractionated heparin, or as the starting material for the synthesis of low-molecular-weight heparins (LMWH).

Heparin can be extracted from various tissues in pigs, cattle, and sheep, including the lung, intestine, or skin. Although the European Pharmacopoeia (Ph. Eur) [1] and U.S. Pharmacopeia (USP) [2] specify that only heparin derived from porcine intestinal mucosa can be used as the starting material for manufacturing LMWH, bovine or ovine heparins can be found in industrial quantities necessitating the use of appropriate analytical methods to detect a potential contamination. At the end of 2007, the adulteration of heparin by oversulfated chondroitin sulfate (OSCS) resulted in several fatal issues during in its clinical use [3], further highlighting the importance of the quality control of heparin. The objectives of this quality control should address several issues: it must first assess the anticoagulant activities, enable discriminant structural characterization of the product, and detect potential contamination. However the analysis of heparin is very complex because heparin is a mixture of polydisperse heteropolymers with a high molecular weight (10,000–20,000 Da). No single method has sufficiently high-resolving power to characterize its structure, making the use of a set of orthogonal methods necessary; this is also true in the case of LMWH. The Ph. Eur. and USP monographs require ^{1}H NMR spectroscopy, strong anion exchange (SAX) chromatography, and molecular weight characterization by gel permeation chromatography; yet, these methods do not allow a precise structural assessment of the heteropolymer composition.

The use of enzyme lyases such as heparinases to depolymerize the glycan into building blocks that can be fully characterized by chromatography, is a technique of major interest as it is simple and informative. Although it remains far from being able to illustrate the true complexity of heparin or LMWH, this technique can provide a first insight into the heparin source (crude or pure heparin), its composition consistency, the animal species of origin, and potential contamination. Moreover, it can detect structural modifications to heparin and can therefore be used to monitor the chemical transformations that occur during the heparin manufacturing process. Currently, although this method indirectly appears in the USP and Ph. Eur. enoxaparin monographs for the control of 1,6-anhydro derivatives [5,6,14], it is not included in the current heparin monographs, nor is it recommended in the FDA guideline [4]. Previous publications have referred to this

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Abbreviations for saccharide units and chemical modifications

U	uronic acid
IdoA	L-iduronic acid
GlcA	D-glucuronic acid
ΔU	4,5-unsaturated uronic acid, e.g. ΔGlcA
GlcN	D-glucosamine
NS	*N*-sulfate
NAc	*N*-acetyl
2S	2-O-sulfate
6S	6-O-sulfate
GalA	D-galacturonic acid

Structural symbols

ΔIVa	ΔU-GlcNAc
ΔIVs	ΔU-GlcNS
ΔIIa	ΔU-GlcNAc,6S
ΔIIIa	ΔU2S-GlcNAc
ΔIIs	ΔU-GlcNS,6S
ΔIIIs	ΔU2S-GlcNS
ΔIa	ΔU2S-GlcNAc,6S
ΔIs	ΔU2S-GlcNS,6S
ΔIVs_{gal}	ΔGalA-GlcNS
ΔIIs_{gal}	ΔGalA-GlcNS,6S
IIs_{glu}	GlcA-GlcNS,6S
$IIIs_{id}$	IdoA2S-GlcNS
IVs_{glu}	GlcA-GlcNS
IIa_{id}	IdoA-GlcNAc,6S
$IIIa_{id}$	IdoA2S-GlcNAc
Ia_{id}	IdoA2S-GlcNAc,6S
Is_{id}	IdoA 2S-GlcNS,6S

The iduronic (id) or glucuronic (glu) structure of uronic acids is indicated for oligosaccharides, e.g. ΔIs–$IIIs_{id}$. Underlined disaccharides have a 3-O-sulfated glucosamine, e.g. $\underline{IIs}_{glu}$ (GlcA-GlcNS,3S,6S).

method [7] but lack precision. It therefore appears important to provide precise and comprehensive description of this important and rather simple analytical tool.

The cleavage of the heparin glycosidic linkages by heparinases generates, after β-elimination, a new oligosaccharide with a Δ-4-5 unsaturated uronic acid at its non-reducing side. When the 3 heparinases are applied together, 8 major unsaturated disaccharides [22,23] are formed, with varying sulfation patterns in position C-2 and C-6 and on the glucosamine either *N*-sulfated or *N*-acetylated. In addition, longer oligosaccharides resistant to heparinases, such as 3-O sulfated tetrasaccharides, are generated [9–11]. Similarly, glycoserine tetrasaccharides, residues of the heparin–glycoprotein linkage and also heparinase-resistant are observed [12,13]. Some methods for the quantitative analysis of heparin and LMWH digests have already been developed [15–19]. However these have important limitations such as insufficient resolution, incomplete elution of the components mixture, or fragmentary achievement of the quantitative aspect. They also include fluorescent tagging on the saccharide reducing-end to increase detectability, which is justified for low-concentration biological samples but is inappropriate and a source of potential error for heparin samples where the UV absorbance of unsaturated acids at 232 nm is sufficient for detection. Moreover, the labeling efficiency of substrates is often variable (e.g. null for 1,6-anhydro derivatives) and side reactions can occur, further increasing the complexity of the sample analysis.

Quantification methodology using external standards is hard to implement due to the number of components, the time necessary to purify standards, and the expected precision associated with the purity of these standards. The most suitable alternative for quantification is internal standardization. Two previously published quantitative methods use ΔIp (ΔU2S-GlcNCOEt,6S) as internal standard and fluorescent tagging by 2-aminoacridone [16,18]. In the first one [16], capillary electrophoresis is used, but the selectivity is insufficient to resolve all components of heparin digests. In the second one [18], the separation is performed by reversed-phase chromatography, identification by mass spectrometry (MS) and quantification by double UV detection. A method of quantitation by LC–MS [19] using isotopic internal standards chemo-enzymatically prepared is also described, but this method cannot be applied in a control laboratory.

In the method described in this paper, the quantification is based on the data driven consensus assumption that molar extinction coefficient at 232 nm for Δ-4-5 unsaturated oligosaccharides are constant [8]. However, to apply this rule to heparin digests, it is necessary to identify every components of the digest to assess their molecular weights. We thus describe here a comprehensive method to identify and quantify the building blocks of heparin generated by heparinase digestion. All natural components (disaccharides and tetrasaccharides) and other major features generated by the heparin purification process are reported.

2. Material and methods

2.1. Materials

All heparin samples used in this study were extracted from porcine mucosa. Purified heparin samples were obtained from different sources available on the industrial market. All enzyme lyases from *Flavobacterium heparinum* (heparinase I [EC 4.2.2.7], heparinase II [no EC number], and heparinase III [EC 4.2.2.8]) were obtained from Grampian Enzymes (Aberdeen, UK). All other reagents and chemicals were of the highest quality available. Water was purified using a Millipore Milli-Q purification system.

2.2. Enzymatic digestion

Heparin digestion (20 μL of a 20 mg/mL solution in water) was performed at room temperature for 48 h, in a total volume of 160 μL containing 20 μL heparinase I, II, and III mixture (0.5 IU/mL of each heparinase in a potassium phosphate buffer pH 7.0 [10 mM KH_2PO_4 and 0.2 mg/mL of bovine serum albumin(BSA)]) and 120 μL of 100 mM sodium acetate buffer (pH 7.0) containing 2 mM of $Ca(OAc)_2$ and 0.1 mg/mL BSA.

2.3. Analysis of heparin digests by SAX chromatography

Exhaustively-digested heparin (4–10 μL) was injected onto a Spherisorb-SAX chromatography column (250 × 3.2 mm, 5 μm, column temperature 50 °C, Waters, France). Mobile phase A was 1.8 mM NaH_2PO_4 at pH 3.0 and mobile phase B was an aqueous solution of 1.8 mM NaH_2PO_4 with 1 M $NaClO_4$ adjusted to pH 3.0. A linear gradient of mobile phase B ($t_{0\ min}$ 3%; $t_{20\ min}$ 35%; $t_{50\ min}$ 100%) was applied with a flow rate of 0.45 mL/min. Double UV detection was performed at 232 nm and 202–247 nm. *N*-acetylated oligosaccharide selective signal (202–242 nm) is the result of the subtraction of the 202 nm wavelength signal from the 247 nm reference signal, as previously described [10].

2.4. Analysis of heparin digests by LC/MS

Heparin digests were injected on ion-pair LC/MS chromatography using experimental conditions derived from those described by Doneanu et al. [20]. Briefly, Acquity UPLC BEH C18 column