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Analytical comparison of a US generic enoxaparin with the originator product: The focus on comparative assessment of antithrombin-binding components

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ABSTRACT

Enoxaparin sodium, a low-molecular-weight heparin (LMWH) prepared from porcine intestinal heparin, is widely used for the prevention and treatment of venous thromboembolism. The antithrombotic activity of heparin is mediated mainly through its activation of antithrombin (AT) and subsequent inhibition of coagulation factors. Heparin is a complex heteropolymer and the sulfation pattern of its alternating uronic acid and glucosamine sugar units is a major factor influencing its biological activity. The manufacturing process itself is associated with the introduction of exogenous microheterogeneities that may further affect its biological efficacy. This is important since enoxaparin is prepared by depolymerizing the heparin with the aim of optimizing its biological activity and safety. Changes during its manufacture could thus affect its biological activity and safety. The current study was performed to assess potential differences between the originator enoxaparin and a new generic enoxaparin commercialized by Teva. Heparinase digestion, AT affinity chromatography, gel permeation chromatography, anion exchange chromatography, and nuclear magnetic resonance methodologies were used. The results indicated differences in oligosaccharides related to the cleavage selectivity around the heparin AT-binding sequences of the Teva Enoxaparin Sodium Injection, USP and the originator Sanofi enoxaparin. These differences influence the strength of the AT-binding affinity of the individual oligosaccharides, their ability to activate AT and, therefore, the inhibitory potency on the proteases of the coagulation cascade. This study, together with other published analytical reports, describes specific compositional differences between generics and originator LWMHs. However, it is yet to be established whether such variations might have any clinical relevance.

1. Introduction

Enoxaparin sodium, a low-molecular-weight heparin (LWMH) prepared from United States Pharmacopeia (USP)-grade heparin sodium, is generally used as an anticoagulant for the prevention and treatment of venous thromboembolism. Heparin is a highly sulfated linear heteropolymer composed of alternating units of uronic acids (α-L-iduronic acid or β-D-glucuronic acid) and D-glucosamines. The latter sugar residues may be *N*-sulfated, *N*-acetylated, or *O*-sulfated at position 6, and less frequently, at position 3. The uronic-acid residues can be *O*-sulfated at position 2, although this is far more unusual for glucuronic acid than for iduronic acid. The sulfation pattern responsible for these microheterogeneities in heparin has an impact on its pleiotropic effects and interaction with hundreds of proteins and enzymes [1].

The antithrombotic activity of heparin is mediated mainly through the activation of antithrombin (AT) [2] and the formation of ternary complexes with coagulation factors, principally factors Xa and IIa [3]. Rare 3-*O* sulfation moieties present on the D-glucosamine residues are responsible for the specific binding of heparin to AT [4,5]. The endogenous structure of heparin can, however, be partially denatured by chemical side reactions that may

Abbreviations: AT, antithrombin; BSA, bovine serum albumin; CTA, cetyltrimethylammonium; Gal, galactose; GlcA, glucuronic acid; LMWH, low-molecular-weight heparin; NMR, nuclear magnetic resonance; SAX, strong anion exchange; USP, United States Pharmacopeia.

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Nomenclature

U	Uronic acid
IdoA	L-Iduronic acid: α-L–Idopyranosyluronic acid
ΔU	4,5-Unsaturated uronic acid, e.g. ΔGlcA: 4-deoxy-α-L-*threo*-hex-enepyranosyluronic acid
ΔU_{2S}	Trisulfated disaccharide
2S	2-*O*-Sulfate
3S	3-*O*-Sulfate
6S	6-*O*-Sulfate

Structural Symbols

ΔIVa	ΔU-GlcNAc
ΔIVs	ΔU-GlcNS
ΔIIa	ΔU-GlcNAc,6S
ΔIIIa	ΔU 2S-GlcNAc
ΔIIs	ΔU-GlcNS,6S
ΔIIIs	ΔU 2S-GlcNS
ΔIa	ΔU 2S-GlcNAc,6S
ΔIs	ΔU 2S-GlcNS,6S
ΔIVs_{gal}	ΔGalA-GlcNS
ΔIIs_{gal}	ΔGalA-GlcNS,6S

The iduronic (id) or glucuronic (glu) structure of uronic acids is indicated for oligosaccharides. Underlined disaccharides have a 3-O-sulfated glucosamine

ΔIIs	ΔU-GlcNS,3S,6S
ΔIs	ΔU2S-GlcNS,3S,6S
IIs_{glu}	GlcA-GlcNS,3S,6S
$\Delta IIa\text{-}IIs_{glu}$	ΔU-GlcNAc,6S- glcA-GlcNS,3S,6S
ΔU 2S	Percentage of oligosaccharides starting with 2-*O* sulfated uronic acid
ΔU	Percentage of oligosaccharides starting with uronic acid without 2-*O* sulfation
NHAc 1	Acetyl group at the non-reducing end, on the first disaccharide building block
NHAc cent	Acetyl group inside the oligosaccharide chain
NAc nbr/chain	Number of acetyl groups per oligosaccharide chain

occur during the manufacturing process. Indeed, several chemical steps required during the preparation of pure USP-grade heparin introduce new, exogenous microheterogeneities that can act as a fingerprint for the origin of the heparin [6].

Enoxaparin is prepared by the chemical depolymerization of the heparin heteropolymer with the aim of optimizing its biological activity and improving its efficacy and safety profile. The enoxaparin manufacturing process described in our previous work modified the endogenous backbone through the introduction of new moieties, such as 1,6 anhydro bicyclic rings [7,8]. Briefly, the polysaccharide was esterified using benzyl ester and then depolymerized by sodium hydroxide under appropriate operating conditions. The overall reaction conditions and the nature of the depolymerizing base determine the structure and amounts of the specific sequences generated, as well as the biological activity profile of the end product. Significant changes such as the choice of the base, e.g. a phosphazene base, were shown to change the pharmacologic activity of the resulting product [9].

The first two US-approved [10] generic enoxaparins produced by Sandoz and Amphastar were compared with the Sanofi originator enoxaparin in 2015. Two studies, the first based on high-performance liquid chromatography (HPLC) [11] and the second on nuclear magnetic resonance (NMR) [12], indicated that the generic enoxaparins showed specific differences when compared to the originator. The use of compositional building block analysis [6,13] was sufficient to distinguish generic enoxaparins from the originator.

To study the Enoxaparin Sodium injection, USP by Amphastar and Sandoz, methodologies and analytical procedures such as AT affinity chromatography, gel permeation chromatography (GPC), and cetyltrimethylammonium–strong anion exchange (CTA-SAX) chromatography were used [11]. The structural differences between the generic and originator compounds were more pronounced than would have been expected in any batch-to-batch variations likely to occur during manufacture of biological products. These differences were statistically significant and permitted to distinguish between the generic enoxaparins produced by the two manufacturers.

Other methods of analysis, the majority based on a single chromatographic separation followed by mass spectroscopy analysis [14–18], have been proposed as suitable for the characterization of LMWHs, however, these methods may not be sufficient since they do not include any AT affinity chromatography step, which supplements the process with a key additional orthogonal resolving power.

In February 2015, an Enoxaparin Sodium Injection, USP Teva was launched in the US market. The current study compares the structure of the Teva generic enoxaparin with the originator Sanofi compound using identical methodologies as those used previously to study the Amphastar and Sandoz generic enoxaparins [11].

Our study also includes data on semuloparin. Semuloparin is an experimental ultra-LMWH. Its development was stopped in 2012. The inclusion of the semuloparin data is not intended as a comparison with the enoxaparin compounds. Instead semuloparin is used as an analytical reference, as extensive structural studies conducted with semuloparin identified many AT binding oligosaccharides. Therefore, semuloparin data are intended as a HPLC reference for identification of oligosaccharides and a NMR reference for detection of important characteristic signals.

2. Materials and methods

2.1. Materials

Two samples of the Enoxaparin Sodium Injection, USP Teva manufactured by Italfarmaco, AB 14023, expiry date 12–2017 (T1) and AB 15027, expiry date 02-2018 (T2), were purchased in the USA as prefilled syringes. A batch of Sanofi enoxaparin, EEA 1412E (S1), in powder form was used as the reference enoxaparin for this comparability exercise (S1 was one of the batches used in the previous Amphastar and Sandoz study [11]). Semuloparin was also obtained from Sanofi (Vitry-sur-Seine, France). Heparinase I (EC 4.2.7), heparinase II (no EC number), and heparinase III (EC 4.2.2.8) from *Flavobacterium heparinum* were obtained from Grampian Enzymes (Orkney, UK). All other reagents and chemicals were of the highest quality available. Water was purified using a Millipore Milli-Q purification system (Darmstadt, Germany).

2.2. Exhaustive depolymerization using the heparinase mixture (disaccharide building block analysis)

Enoxaparin samples were digested in the heparinase mixture as described previously [6,11]. Briefly, enoxaparin samples (20 μl of a 20 mg/ml solution in water) were digested at room temperature for 48 h in a total volume of 170 μl containing 20 μl of a mixture of heparinase I, II, and III. Each heparinase was at 0.5 IU/ml in a potassium phosphate buffer (pH 7.0; 10 mM KH_2PO_4 and