



Commercial Low Molecular Weight Heparins — Patent Ecosystem and Technology Paradigm for Quality Characterization

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Abstract

Heparin is a subject of ever-growing interest for laboratory researchers and pharmaceutical industry. One of the driving factors is its critical life-saving drug status, which during the COVID-19 pandemic has assumed a central role in disease treatment and/or prevention. Apart, heparin is one amongst few drugs enjoying a “demand constant” status. In 2020, heparin market size was valued to US\$6.5 bn., and given the ongoing stability in the COVID-19 health crisis, it is expected to reach US\$11.43 bn. by 2027 with yearly growth rate momentum (CAGR) of 3.9% during the forecast period (Pepi et al., *Mol Cell Proteomics* 20:100,025, 2021). As patent is a limited monopoly, every year, many patents on low molecular weight heparin (LMWH; a chemically or enzymatically degraded product of unfractionated heparin) are losing market exclusivity worldwide, inviting the generic/biosimilar drug manufacturers to capture market share with cheaper drug products. By tracking patent expiration, drugs in patent litigation, regulatory setbacks for innovator companies (such as those seeking data exclusivity or patent term extension), or other unexpected events affecting market demand and competition, generics can make investment decisions in manufacturing off-patent LMWH drug products of commercial significance. However, given the US Food and Drug Administration (FDA), European Medicine Agency (EMA), Drug Regulatory Authority of Pakistan (DRAP), and other regulatory authorities scientifically rigorous standards for generic/biosimilar LMWH drug products marketing approval, the market is secured and momentous for drug makers that could demonstrate through scientific and clinical dataset that the generic/biosimilar LMWH drug product is of the same quality and purity as the innovator drug product. This study presents an overview of the patent landscape of commercially available LMWHs and advanced analytical techniques for their structural and biochemical characterization for quality control and quality assurance during manufacturing and post-marketing. The study also covers FDA, EMA, Health Canada, and DRAP’s current approaches to evaluating the generic/biosimilar LMWH drug products for quality, safety including immunogenicity, and efficacy.

Keywords Biosimilars · Enoxaparin · Generics · Heparin · Low molecular weight heparin · Patent · Regulatory authority

Highlights

- Underlines LMWHs’ significance as a life-saving drug and increase in market demand during COVID-19 crisis.
- Accentuates impact of loss of exclusivity over LMWH drug products of commercial significance and increase in opportunities for market entry with low-cost generic drug products.
- Elucidates scientifically rigorous standards for generic drug makers to get marketing approval of generic LMWH drug products to prove bioequivalence (same quality, clinical efficacy, safety including immunogenicity as the innovator drug product).
- Presents overview of patent landscape of commercially significant LMWHs and analytical techniques developed for quality characterization (structural and biochemical).
- Describes current approaches on analytical characterization and challenges for evaluating and maintaining control over quality and consistency in reproduction of generic LMWH drug products.

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Introduction

Heparin (also known as “standard heparin”) is a highly sulfated glycosaminoglycan (GAG) of animal origin having weight-average molecular weight (M_w) of ~ 12,000 Da corresponding to ~ 40 saccharide units [1, 2]. Since 1939, it has been used as a pharmaceutical intervention in a number of diseases including thrombosis, which is the formation of thrombus (blood clot) that sometimes may lead to pulmonary embolism, a potentially life-threatening condition [3]. Heparin structure is characterized by (i) repeating, 1 → 4-glycosidically linked disaccharide building blocks composed of one uronic acid (either glucuronic acid (GlcA) or iduronic acid (IdoA) and one glucosamine moiety (GlcN) that is either N-sulfated or N-acetylated; (ii) various substitution patterns of sulfation in the form of O-sulfation at

the C2 position of IdoA, and C3 and C6 position of GlcN, or in the form of N-sulfation at the C2 position of GlcN [4]; (iii) a unique pentasaccharide sequence — GlcNS/Ac6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S — distributed within the heparin polymer, having high affinity for binding to antithrombin III (ATIII); and (iv) a partially characterized octadecasaccharide sequence that together with pentasaccharide sequence activate ATIII to inhibit coagulation factor Xa and factor II (prothrombin) [5] (Fig. 1A).

Heparin also has a linkage tetrasaccharide sequence — GlcA-Gal-Gal-Xyl-Ser — at its reducing end that covalently link heparin to serine core of proteoglycan during synthesis and elongation [5] (Fig. 1B). The disaccharide sequence — IdoA2S-GlcNS6S — is the major repeating structural unit and constitutes the highly sulfated region of heparin [5].

Heparin exerts its anti-coagulation activity (anti-IIa activity) through high-affinity binding to and activation of ATIII, which is a protease inhibitor including thrombin (factor IIa) and factor Xa [6]. Commercial preparations of heparin are available from marketing companies including the following: Abbott, Organon, Riker, Invenex, Baxter, Calbiochem, Sigma Tau, Changzhou Qianhong CQ Biopharma, Nanjing King Friend, and Upjohn. A major adverse effect associated

with heparin is the heparin-induced thrombocytopenia (HIT). Several studies have concluded heterogeneity in the GAG chains, their length, molecular weight (MW) distribution, and degree of sulfation as key factors for heparin varying binding tendency with other components in the blood plasma and consequent neutralization [1, 7]. Parallel studies regard non-template-driven biosynthesis, the principal cause of complexity, and polydispersity in the GAG chains [1, 8]. Consequent upon Andersson et al.'s [9] findings that LMWHs of different MWs influence the coagulation process differently, several heparin fragments with more selective and predictable pharmacological action had been developed [2] and are the subject of proprietary rights in the USA, Europe, and other countries where substantive patent laws are practiced and enforced.

The very first patent on LMWH dalteparin by Lindahl et al. [10] was expired in 2000, opening-up the market for low costs generic/biosimilar competition, and in parallel fuelling the quest for further innovations and patent protection. As more innovative LMWH products went generic, analytical characterization, control of quality, and regulations including consistency in heparin-derived products supply chains become more and more challenging. Several

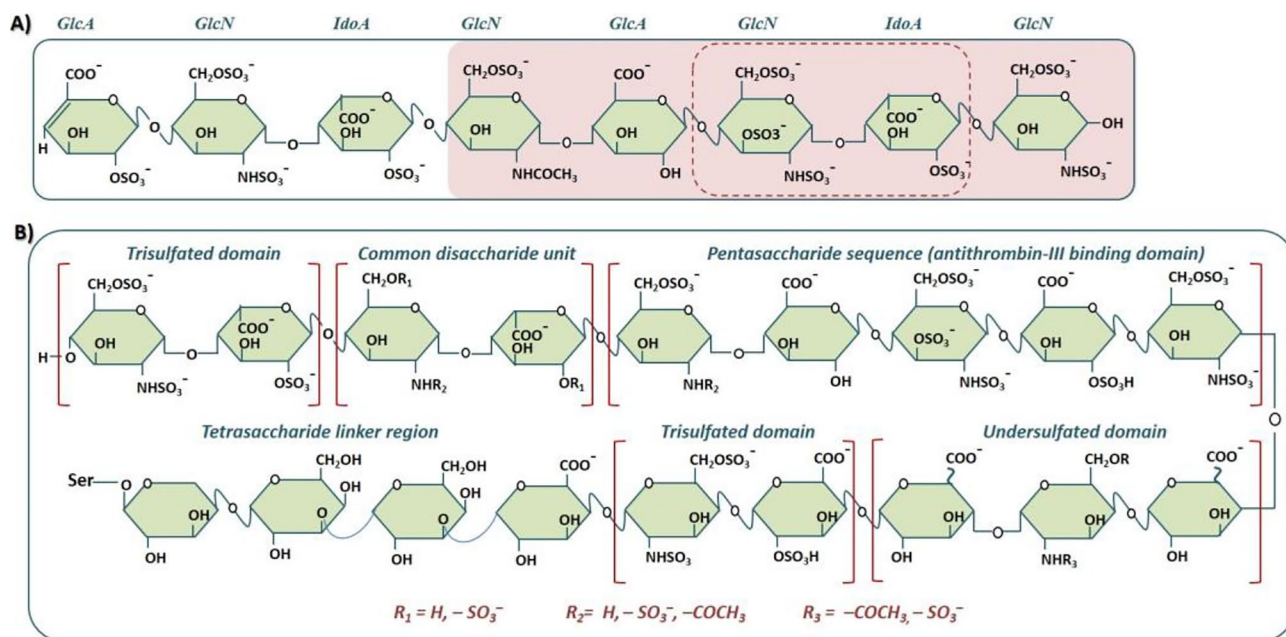


Fig. 1 **A** Heparin with a representative polysaccharide containing four disaccharide building blocks composed of one uronic acid (UA) and one glucosamine (GlcN) moiety. The disaccharide sequence — GlcNS3S6S-IdoA2S — in the dashed frame (FG) constitutes the highly sulfated region and major repeating structural unit within heparin while the block-shaded pink is the pentasaccharide sequence (or antithrombin II-binding domain). One of the two UA residues (iduronic acid, IdoA) present in the pentasaccharide sequence is consistently sulfated at the C-2 position, whereas the hydroxyl groups (OH) at both C-2 and C-3 of the other uronic moiety (glucuronic acid,

GlcA) are unsubstituted. **B** A representative heparin comprising (left to right continuing to lower panel): a trisulfated domain, a common disaccharide unit, a pentasaccharide sequence (antithrombin III-binding domain), a trisulfated domain, and a tetrasaccharide linker region, GlcA-Gal-Gal-Xyl-Ser (source: Wang and Chi [5] Recent advances in mass spectrometry analysis of low molecular weight heparins. *J. Chin. Chem. Lett.*, 2018, 29(1): 11–18; Ekre et al. Use of chemically modified heparin derivatives in sickle cell disease. *US9480702* (2016))

top-down and bottom-up studies on extensive characterization of LMWH complex structural features for quality control and quality assurance and understanding their diversifying role in medicine and pharmacology [11], decrypting their non-template driven biosynthesis to fix heterogeneity and polydispersity in their quality attributes [8], establishing correlation between their structure and biological activity [12], and discovering new clinical applications [13] are available in the published literature. However, amongst these, we find very few reports that specifically highlight the patent stats of commercial LMWH drug products for the purposes of defining the boundaries where the generic drug manufacturers have freedom to operate, discuss advances in the analytical techniques that generic/biosimilar drug manufacturers can utilize for the purposes of establishing that the generic/biosimilar LMWHs contain the same active ingredient as the innovator drug product, and conduct residual uncertainty risk assessment studies to demonstrate the absence of any clinically meaningful differences between the generic and the innovator drug products for the purposes of regulatory approval. This review provides information on this little studied area of technical advancements and commercial value, which the scientific researchers, medical practitioners, and generic/biosimilar drug makers shall find of equal interest.

LMWHs and Production Methods

European Pharmacopoeia (EP) 6.0 (01/2008:0828,2041–2043) defines LMWHs as “salts of sulfated glycosaminoglycan (GAGs) having a weight-average molecular weight (M_w) less than 8000 Da and for which at least 60% of the total mass has a molecular mass less than 8000 Da.” On an average, LMWH has M_w of ~4000 to ~8000 DA corresponding to ~6 to ~12 disaccharide units, which is nearly one third the size of the unfractionated heparin polysaccharide chains [14]. This reduction in molecular size renders the LMWHs more selective and predictable in therapeutic response, high in bioavailability and anticoagulation effect, and practical for subcutaneous infusion [15]. The LMWHs essentially retain the backbone structure of heparin, and only the termini of newly created oligosaccharide chains, modified through chemical or enzymatic depolymerization, display different chemical structures at the reducing and non-reducing ends [5]. These distinctive terminal structures are representative of the characteristics of each type of LMWHs. Nonetheless, a preferential cleavage either towards the highly sulfated region or the undersulfated region of heparin can substantially affect the disaccharide sequence distribution of resulting LMWHs and consequently the binding affinity of the pentasaccharide sequence for AT [16]. As a whole, these structural and compositional differences affect the pharmacological activity of the

LMWH drug product; hence, deeper investigation of LMWH structure and function is critical for pharmaceutical control of quality and consistency in reproduction. So far, approved and commercially available LMWHs in various markets include the following: dalteparin (Fragmin®), enoxaparin (Lovenox®), nadroparin (Fraxodi®), tinzaparin (Innohep®), parnaparin (Fluxum®), bemiparin (Hibor®, Ivor®, Ivorat®, Ivormax®, Badyket®, Zibor®), sevuparin, ardeparin (Normiflo®), reviparin (Clivarin®), and certoparin (Sandoparin®, Embolex®). Table 1 provides production methods and structural characteristics including MWs, signature structures, and degree of sulfation of commercially available LMWHs.

Enoxaparin sodium is the most commonly used LMWH derivative. It is derived from standard heparin through a controlled chemical β -elimination reaction comprising (i) cleaving the polysaccharide backbone and (ii) hydrolyzing the residual esters under alkaline conditions [17]. The most part of oligosaccharide chains in enoxaparin have unsaturated 4-enopyranosuronate residues at their non-reducing ends, and only a minority, accounting 15–25% of the total oligosaccharide chains, has unnatural 1,6-anhydro amino sugar residues at the reducing ends [18]. These unnatural structures at termini constitute the fingerprints or signature structure of enoxaparin [19]. In other instances of LMWH derivatives, such as dalteparin, nadroparin, and reviparin, depolymerization of heparin polysaccharides is carried out through controlled deaminative cleavage using nitrous acid (HNO_2), followed by sodium borohydride (NaBH_4) reduction. This cleavage results in oligosaccharide fragments with reducing ends that are modified to 6-O-sulfo-2,5-anhydro-D-mannitol [20]. In case of tinzaparin sodium, the depolymerization is carried out enzymatically, in particular, with a highly purified heparinase of bacterial origin. Heparinase breaks the heparin saccharide chains between the anomeric carbon (carbonyl carbon) of an N-sulfate-glucosamine and the following uronic acid motif, creating an unsaturated uronic acid structure at its NRE. Ardeparin sodium represents a LMWH that is obtained by oxidative depolymerization of heparin with hydrogen peroxide while parnaparin sodium is obtained by oxidative depolymerization of heparin with Cu^+ [2] and hydrogen peroxide. Both have saturated uronic acid residues at the NRE of the chain. Still, selective oxidation of non-sulfated uronic acid residues in heparin by periodate (NaIO_4), alkaline treatment with quaternary ammonium (NH_4) salt of heparin, deaminative cleavage of heparin polysaccharide using isoamyl nitrite, and chemo-selective depolymerization of heparin in non-aqueous medium (or using 2-tert-butylimino-2-diethylamino-1,3-dimethyl-perhydro-1,2,3-diazaphosphorine (BEMP)) have resulted in different LMWH and ultra-LMWH products, with distinct groups at non-reducing and reducing ends of the oligosaccharide/polysaccharide chains. Summarily, different cleavage methods (enzymatic or chemical) create LMWH products containing different active ingredients with specific

Table 1 Summary of commercial LMWH and ULMW production methods and structural characteristics including molecular weight range, chain end groups, anti-coagulant activity (anti-Xa, anti-IIa), and degree of sulfation

LMWH/ ULMWH (INN)	Brand name	Manufacturer/ marketing company	Mode of depolymer- ization/ method of preparation	MW (Da)	MW (Da)	NRE	RE	Anti- Xa:anti-IIa ratio	Anti-Xa activity (IU/ mg)	Anti-IIa activity (IU/ mg)	Degree of sulfation/ saccharide unit
Dalteparin sodium	Fragmin Boxol FR 860 Tedelparin	Pfizer/Kabi/ Pharmacia- Upjohn (US)	Deaminative cleav- age with nitrous acid (HONO)	5600–6400	6000–5000	2-O-sulfo- α -L- idopyranosuronic acid	6-O-sulfo-2,5- anhydro-D- mannitol	1.9–3.2:1	110–210	35–100	2.0–2.5
Enoxaparin sodium	Lovenox Clexane	Sanofi- Aventis / Rhone- Poulenc/ Aspen Pharma/ Eurofarma Lab	Alkaline β -elim- inative cleavage of benzyl ester of heparin	3500–5500	4500	2-O-sulfo-4- enepyransuronic acid (or 2-sulfated 4,5-unsaturated uronic acid)	2-N-sulfated-D- glucosamine; characterized by 1,6-anhydro ring structure/2-N,6- O-disulfo-D- glucosamine	3.3–5.3:1	100–210	20–35	~2.0
Tinzaparin sodium	Innohep Logiparin	LEO Pharma/ Novo Nordisk/ Braun/ DuPont/ Pharmion	β -elimnative cleavage by hepari- nase/	5500 to 7500	6500	2-O-sulfo-4- enepyransuronic acid (or 2-sulfated 4,5-unsaturated uronic acid)	2-N,6-O-disulfo-D- glucosamine	1.5–2.5:1	70–120	45–50	2.66
Nadroparin calcium	CY-216 Fraxiparin Seleparina	Sanofi- Winthrop/ Choay/ Aspen/Ital- farmaco	Deaminative cleav- age with nitrous acid (HONO)	4200 to 5500	4300	2-O-sulfo- α -L- idopyranosuronic acid	6-O-sulfo-2,5- anhydro-D- mannitol	2.5–4.0:1	95–130	27–37	2.0–2.5
Bemiparin sodium	Badyket Ivor Zibor Hibor Beparine	Laboratorios Farmacia- ceuticos Rovi S.A. /Sigma Tau/UCB / Biological Evans	Alkaline treat- ment with quaternary ammonium (NH ₄) salt of heparin	3000 to 4200	3600	2-O-sulfo-4- enepyransuronic acid	2-N,6-O-disulfo-D- glucosamine	8.0:1	80–100	10–12.5	About 2 (WHO)

Table 1 (continued)

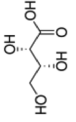
LMWH/ ULMWH (INN)	Brand name	Manufacturer/ marketing company	Mode of depolymerization/ method of preparation	MW (Da)	MW (Da)	NRE	RE	Anti- Xa:anti-IIa ratio	Anti-Xa activity (IU/ mg)	Anti-IIa activity (IU/ mg)	Degree of sulfation/ saccharide unit
Sevuparin (DF02)	N/A	Modus Therapeutics AB/ Dilafor AB	Selective oxidation of non- sulfated uronic acid residues in heparin by periodate	6500 to 9500	5000	2-N,6-O-disulfo-D- glucosamine	Glucosamine bound to a "remnant" residue (remnant = D-threonic acid)* 	1.5:1	< 10	< 10	2.4
Parnaparin sodium	Alpha LMWH 86-02 Fluxum Minidalon OP-21-23	Alfa Wassermann SpA	Oxidative depolymerization with cupric ions (Cu ²⁺) and hydrogen peroxide (H ₂ O ₂)	4500 to 5000	5000	2-O-sulfo-α-L- idopyranosuronic acid	2-N,6-O-disulfo-D- glucosamine	1.5-3.0:1	75-110	25-30	2.15
Reviparin sodium	Clivarin LU 473,111	Knoll AG/ Abbott GmbH	Deaminative cleavage with nitrous acid (HONO)	3400 to 4650	3900	2-O-sulfo-α-L- idopyranosuronic acid	6-O-sulfo-2,5- anhydro-D- mannitol	4.2:1	124	29	2.0-2.6
Ardeparin sodium (withdrawn from US market in 2000) (www. drugs.com)	Normiflo RD 11,885 WY- 90493-RD	Wyeth- Ayers/ Hepar Industries/ Pfizer	Oxidative depolymerization with hydrogen peroxide (H ₂ O ₂)	2000 to 15,000	5300-6500	2-O-sulfo-α-L- idopyranosuronic acid or saturated uronic acid	2-N-acetyl-6-O- sulfo-D- glucosamine	1.8:1	95-145	45-75	2.0-2.7
Certoparin sodium	Alphaparin Sandoparin Mono- Embolex NM, Troparin	Novartis/ Sandoz/ Aspen in EU	Deaminative cleavage with isosoyl nitrite	4200-6200	5200	2-O-sulfo-α-L- idopyranosuronic acid	6-O-sulfo-2,5- anhydro-D- mannitol	1.5-2.5:1	80-120	30-35	2.0-2.5

Table 1 (continued)

LMWH/ ULMWH (INN)	Brand name	Manufacturer/ marketing company	Mode of depolymer- ization/ method of preparation	MW (Da)	MW (Da)	NRE	RE	Anti- Xa:anti-IIa ratio	Anti-Xa activity (IU/ mg)	Anti-IIa activity (IU/ saccharide unit)	Degree of sulfation/ saccharide unit
Fondaparinux sodium	Arixtra	Aspen	Chemically synthesized by O-sulfation-hydrogenation-N-sulfation of pentasaccharide	1500–3000	1728	2-N,6-O-disulfo-D-glucosamine	Methyl-2-N,6-O-disulfo-D-glucosamine (or 6-O-sulfo-2-(sulfoamino)- α -D-glucopyranoside)	Specific FXa inhibitor*	930	0	1.6
AVE5026* (A derivative of enoxaparin)	Semuloparin	Sanofi-Aventis	Chemoselective depolymerization by BEMP following β -elimination	2000 to 3000	2400	2-O-sulfo-4-enopyranosuronic acid (or 4,5 unsaturated uronic acid or 4-enopyranosyluronate)	-	> 30:1	150–200	0.2 or < 5	2.2
RO-14 (A derivative of bempirin)	N/A	Laboratorios Farmaceuticos Rovi S.A	Chemoselective depolymerization of heparin in non-aqueous medium following β -elimination	1800 to 3000	2200	2-O-sulfo- α -L-idopyranosuronic acid (or 4-enopyranosyluronate)	2-N,6-O-disulfo-D-glucosamine	9.7:1	80–140	≤ 10	2.0

Sources: Min Qiu, Shengjie Huang, Chuanhong Luo, Zhenfeng Wu, Binzhu Liang, Haozhou Huang, Zhimin Ci, Dingkun Zhang, Li Han, Junzhi Lin, Pharmacological and clinical application of heparin progress: An essential drug for modern medicine, *Biomedicine & Pharmacotherapy* 139 (2021) 111,561; Yan Y, Ji Y, Su N, Mei X, Wang Y, Du S, Zhu W, Zhang C, Lu Y, Xing XH. Non-anticoagulant effects of low molecular weight heparins in inflammatory disorders: A review. *Carbohydr Polym*. 2017 Mar 15;160:71–81. <https://doi.org/10.1016/j.carbpol.2016.12.037>. Epub 2016 Dec 21. PMID: 28,115,102; Hao, C., Sun, M., Wang, H., Zhang, L., & Wang, W. (2019). Low molecular weight heparins and their clinical applications. *Progress in Molecular Biology and Translational Science*. <https://doi.org/10.1016/b978-0-12-815111-1.1538-7836>; Akhtar F, Wan X, Wu G, Kesse S, He S. *Low-Molecular-Weight Heparins: Reduced Size Particulate Systems for Improved Therapeutic Outcomes. Molecules*. 2018 Jul 18;23(7):1757. <https://doi.org/10.3390/molecules23071757>. PMID: 30,021,958; PMCID: PMC6100363; Lühn S, Grimm JC, Alban S. Simple and rapid quality control of sulfated glycans by a fluorescence sensor assay—exemplarily developed for the sulfated polysaccharides from red algae *Delesseria sanguinea*. *Mar Drugs*. 2014 Apr 10;12(4):2205–27. <https://doi.org/10.3390/md12042205>. PMID: 24,727,392; PMCID: PMC4012468; Bisto A, Urso E, Guerrini M, de Wit P, Torri G, Naggi A. *Structural Characterization of the Low-Molecular-Weight Heparin Dalteparin by Combining Different Analytical Strategies. Molecules*. 2017 Jun 24;22(7):1051. <https://doi.org/10.3390/molecules22071051>. PMID: 28,672,818; PMCID: PMC6152074; *Gerotziadas GT, Petropoulou AD, Verdy E, Sanama MM, Elalamy I. Effect of the anti-factor Xa and anti-factor IIa activities of low-molecular-weight heparins upon the phases of thrombin generation. *J Thromb Haemost*. 2007 May;5(5):955–62. <https://doi.org/10.1111/j.1538-7836.2007.02477.x>

BEMP 2-tert-butylimino-2-diethylamino-1,3-dimethylperhydro-1,2,3-diazaphosphorin

* Source: WO2009007224A1; NIH — National Center for Advancing Translational Sciences, Inxight; <https://drugs.ncats.io/substance/V72OT3K191>, accessed: June 30, 2022

MWs, disaccharide building block compositions, and distribution of their sequences in the oligosaccharide chains, as well as distinct structural signatures and hence are not substitutable (see Fig. 2).

Commercial LMWH Patent Ecosystem

Globally, there are more than 5000 patents covering production and/or purification methods, new therapeutic applications, dosing regimens, new formulations/mixtures, and advanced analytical approaches for structural characterization of LMWHs including the most widely used enoxaparin/enoxaparin sodium [21], but our focus here is to discuss some of the key patents (see Table 2) on the commercially available LMWHs and ultra-low molecular weight heparins (ULMWHs) so that manufacturers of generic/biosimilar LMWHs may know the boundaries of competitor's IP portfolio and their freedom-to-operate in the relevant IP ecosystem.

The first European patent EP0014184A2 (~ US Patent No. 4303651A1; Lindahl et al.) describing LMWH — dalteparin sodium — was granted to Kabi AB in 1989 and is currently being marketed by Pfizer Health AB under the brand name Fragmin®. Going into the very details of this significant innovation, in 1980, Lindahl et al. [10] prepared a new heparin fragment through controlled nitrous acid depolymerization of heparin sodium in aqueous medium. The group suggested several other ways to preparing the new heparin fragment such as periodate oxidation, partial depolymerization with heparinase, partial depolymerization by esterification of carboxylic groups and subsequent alkaline R-elimination, and partial depolymerization by partial N-desulfation and subsequent deamination with nitrous acid. With very weak inhibitory effect on thrombin (factor IIa), the new heparin fragments had a very strong inhibitory effect on activated coagulation factor Xa that assumed a central position in the middle of the coagulation cascade. Structural analysis of the Lindahl's LMWH revealed the same disaccharide unit (i.e., L-iduronosyl-2-O-sulfate-(1)-N-sulfo-D-glucosamine-6-O-sulfate) as the dominating component as in the starting material but with enhanced amount of unsulfated iduronic acid from 6 to 16% relative to the starting material. The US patent on dalteparin sodium was expired in 2005, but the non-patent data exclusivity still authorizes Pfizer to maintain their exclusive hold on the market until May 16, 2022. So far, no generic/biosimilar version of dalteparin is available in the USA through authorized channels [22].

The first US patent 5,389,618 (re-issued under No. 38,473) covering the most widely used LMWH — enoxaparin sodium — was expired on February 14, 2012. The patent was one of the two patents, listed in the Orange Book as the FDA's

approved drug product. The second US Patent 4,692,435, was expired on December 24, 2004. The 5-year non-patent data exclusivity for enoxaparin as a compound had long expired in 1998. Enoxaparin sodium is manufactured by Sanofi-Aventis under the brand name Lovenox® (enoxaparin sodium injection in the USA) and Clexane® or Klexane® (in other countries). Sanofi-Aventis obtained enoxaparin sodium through β -eliminative degradation of heparin benzyl ester under alkaline conditions (pH > 10), which they derived from porcine intestinal mucosa. For generic drug product manufacturers, to establish bioequivalence has been a potentially difficult phenomenon [23]. They need high-throughput technology to ensure reliable analytical characterization, pre-clinical evaluation, and immunogenicity assessment of generic/biosimilar LMWHs. Sandoz and Momenta had taken lead to seek approval from US-FDA to sell first generic version of Lovenox® post-patent expiry [24]. Earlier (2003), during the validity term of enoxaparin sodium patent, several companies such as Amphastar Pharmaceuticals and Teva Pharmaceutical Industries made joint efforts to seek US-FDA approval to sell generic versions of Lovenox in the US market. Sanofi filed patent infringement lawsuits against the said generic concerns before the US District Court, California [25]. The Court ruled against Sanofi and invalidated the patent on Lovenox on the ground of "inequitable conduct." Sanofi appealed to the US Court of Appeals for the Federal Circuit that affirmed the District Court decision [26]. More so, the Supreme Court denied the petition for certiorari to Sanofi [27].

In a parallel action before FDA, on February 19, 2003, Sanofi filed a Citizen Petition requiring the authorities to withhold any abbreviated new drug application (ANDA) for generic version of Lovenox until (i) enoxaparin structure is fully characterized; (ii) the manufacturing process used to make generic enoxaparin is demonstrated to be equivalent to the process used by Sanofi for branded enoxaparin, or the ANDA is supported by safety and effectiveness data gathered through clinical trials; and (iii) the generic product contains 1,6-anhydro ring structure at the reducing ends of between 15 and 25% of its polysaccharide chains. Excepting the last request for presence of enoxaparin signature structure in the generic enoxaparin, FDA denied the petition on all other respects. Responding to Sanofi's request for "manufacturing equivalence" of generic enoxaparin, FDA remarked that "to manufacture enoxaparin an ANDA applicant will- i) depolymerize heparin by chemical (alkaline) β -elimination; and ii) adjust the process conditions such that they result in the same active ingredient as Lovenox enoxaparin." FDA extended that to manufacture enoxaparin, the process conditions may be the same as used for the originator enoxaparin but these not necessarily need to [28].

While the US courts and FDA rulings had long cleared the road towards development of generic/biosimilar versions

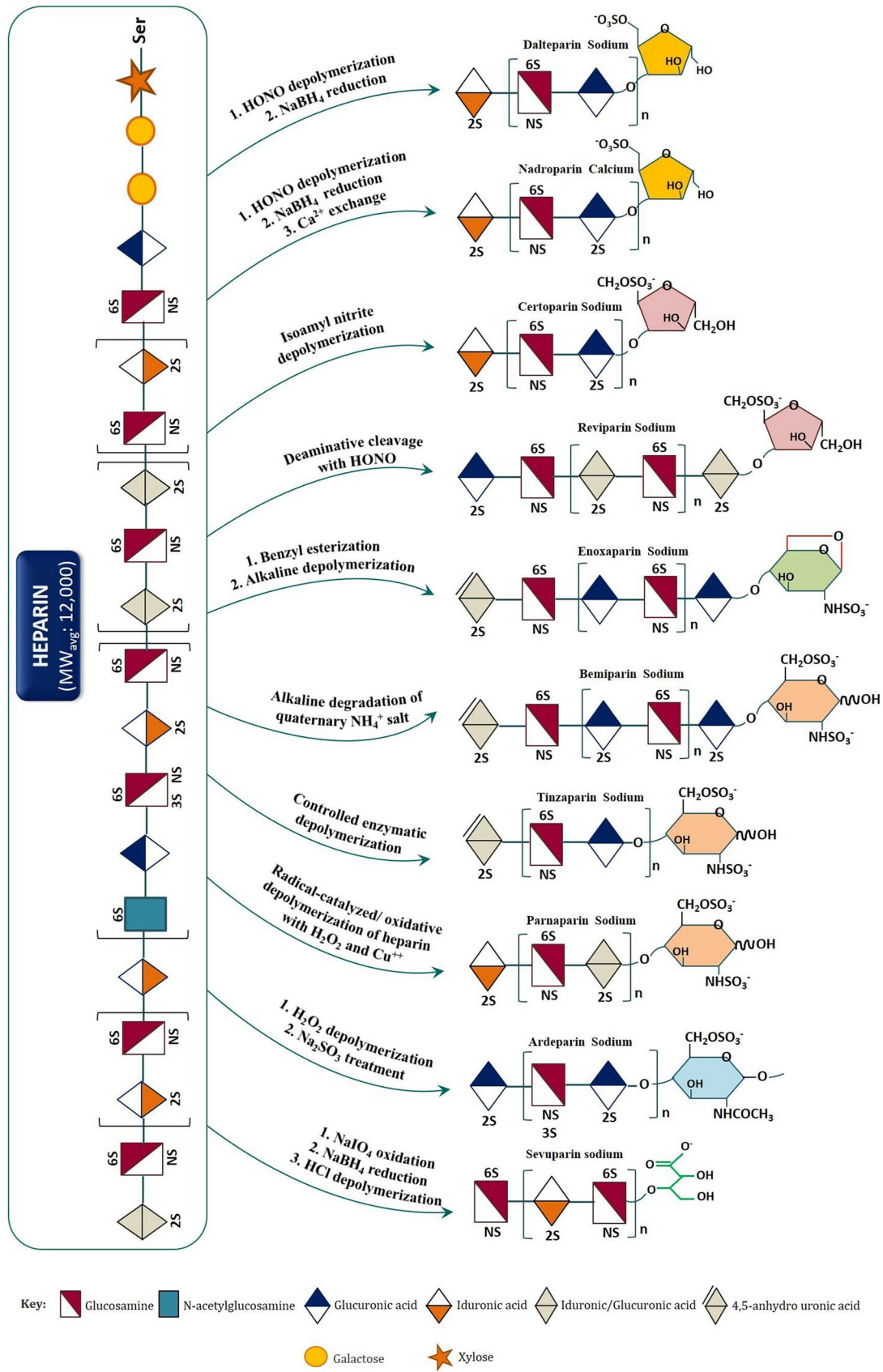


Fig. 2 Scheme of depolymerization (enzymatic or chemical) used to prepare commercially available LMWHs from standard heparin. Signature structures (reducing end groups) are at the extremes right end. Identical groups are shown with same colors. (Sources: Wang Z. and Chi L. Recent advances in mass spectrometry analysis of low molecular weight heparins (2018). *J. Chin. Chem. Lett.*, 29(1): 11–18; Yan Y, Ji Y, Su N, Mei X, Wang Y, Du S, Zhu W, Zhang C, Lu Y, Xing XH (2017). Non-anticoagulant effects of low molecular weight heparins in inflammatory disorders: A review. *Carbohydr Polym.*, 160:71–81. 10.1016/j.carbpol.2016.12.037; Fu L. et al. (2015). Bioengineered heparins and heparan sulfates, *Adv. Drug Deliv. Rev.*, <http://dx.doi.org/10.1016/j.addr.2015.11.002>; Sánchez-Ferrer, C. F. (2010). Bemiparin. *Drugs*, 70, 19–23. 10.2165/1158581-s0-000000000-00000; Patents: US9475888; US10023659; WO2009007224A1; US9012229B2 (Assignee: Hangzhou Jiuyuan Gene Engineering Co., Ltd. and Shanghai Institute of Organic Chemistry, CAS); Web: Japanese Accepted Names (JAN) Name and Structure Database (National Institute of Health Sciences, https://jpub.nihs.go.jp/jan/DetailList_en.aspx?submit=all_alp+Search&keyword=Reviparin+Sodium, accessed: April 20, 2021) Japanese Accepted Names for Pharmaceuticals (JAN Database) jpub.nihs.go.jp)

of off-patent Lovenox® at low costs and for increasing access to patients, nonetheless Sanofi's concerns about therapeutic non-equivalence of the generic/biosimilar enoxaparin drug product due to chemical diversity in the disaccharide building blocks and corresponding distribution of their sequences in the polysaccharide chains with the innovator drug product shall still be remaining there, invigorating the need for developing methods for fine structural characterization and compositional analysis of LMWHs.

Sevuparin sodium (also known as DF02) is another distinctive heparin derivative invented by Ekre et al. and protected by Modus Therapeutics under the US Patent 9480701B2. Ekre et al.'s heparin fragment has a low anticoagulant activity and effectiveness for treating heparin-associated disorders such as malaria. The research group also developed a method for use of DF02 in the treatment of vaso-occlusive crisis in sickle cell disease. As per Ekre's patent description, DF02 is a chemically modified heparin composed of polysaccharide chains having (i) at least 90% of the sulfate groups of source heparin; (b) a reduction in chemically intact saccharide sequences and a reduction in unsulfated iduronic acid and/or glucuronic acid units, when compared to source heparin; and (c) a predominant disaccharide represented by the structure: -IdoA2S-GlcNS6S-OR'- (wherein R' = threonate (C₄H₇O₅) residue). The fragment is derived from heparin sodium and involved selective oxidation of non-sulfated iduronic acid residues in heparin by sodium periodate (NaIO₄), followed by reduction with sodium borohydride (NaBH₄) and treatment with HCl for polymer cleavage at the oxidized site. (Fig. 3 shows schematic reaction scheme of sevuparin preparation.) Structural analysis of DF02 using proton nuclear magnetic resonance

(¹H-NMR) confirmed the absence of unidentified residues or structures that are unexpected in the [¹H-NMR spectrum, hence the product's greater stability. Both the patents stand granted in the name of Modus Therapeutics AB and shall expire on December 19, 2032. Right now, Modus Therapeutics is considering clinical development of sevuparin for the potential treatment of sepsis/septic shock and other inflammatory complications. This substantiates appreciation by leading pharmaceutical companies of the significance of understanding the precise structure of individual components in LMWHs for stringent quality control and improving clinical applications of LMWHs. The first clinical trial for new indications is expected to take start by the end of 2021 [29].

Notwithstanding LMWHs increased bioavailability, more selective activity with respect to activated factor Xa and factor IIa, and predictability in pharmacological action over unfractionated heparin, researchers believe they are still having high anti-IIa activity. To reduce hemorrhage risks, ULMWHs with high anti-Xa activity and zero or very low anti-IIa activity have been developed. US Patent 6,384,021 owned by Laboratorios Farmaceuticos Rovi S.A. discloses ULMWH (RO-14, bemiparin derivative) with anti-Xa activity value 120 IU/mg and anti-Xa/anti-IIa ratio between 15 and 50. Using a different preparation scheme, international patent application WO2002008295A1 by Sanofi-Aventis discloses another ULMWH (AVE5026, semuloparin sodium) that exhibits an anti-Xa activity value 100–150 IU/mg, an anti-IIa activity of 0–10 IU/mg, and anti-Xa/anti-IIa ratio > 10. Through controlling the % age of water (less than 0.6%) during depolymerizing a quaternary ammonium salt of a benzyl ester of heparin in the presence of a base selected from phosphazenes, Sanofi-Aventis developed and disclosed in US Patent 8,003,623 another improved ULMWH (semuloparin sodium) with a mean MW between 1500 and 3000 Da, anti-Xa activity between 161 and 192 IU/mg, an anti-IIa activity < 10 IU/mg, and an anti-Xa/anti-IIa ratio > 30 [30]. Sanofi's new sulfated oligosaccharides are further characterized by the following: 2–26 saccharide units, 4,5-unsaturated uronic acid 2-O-sulfate at one of their ends and a hexasaccharide having high binding affinity for ATIII and an anti-Xa activity > 740 IU/mg (US8003623) [31]. The hexasaccharide represents about 15–25% of the mixture of oligosaccharides. Sanofi also developed and disclosed in WO2012072799A1 new method (amidolytic assays on chromogenic substrate) for in vitro measurement of the biological activity of semuloparin sodium. Another US patent 9,346,894 (~ WO2012140580A1) by Sanofi SA discloses a heparin derivative (a “double site” sulfonated polysaccharides) comprising two ATIII-binding hexasaccharide sequences (Fig. 4). Earlier, Jordan et al. [32] reported

Table 2 Summary of commercial LMWH and ULMWH patent position including the principal claim defining the scope of patent (whether product, process, product-by-process patent) and the date of expiry

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
Dalteparin	EP0014184	Pfizer Health/ Kabi AB	07-01-1980 (08-01-1979 SE)	“Compound/ Heparin fragments, characterized by 14-18 sugar units, the disaccharide unit L-iduronosyl-2-O-sulphate-N-sulpho-D-glucosamine-6-O-sulphate”	18-10-2006
	US4303651	Pfizer Health / Kabi AB	04-01-1980 (08-01-1979 SE)	“Compound/Heparin fragments having 14-18 sugar units, wherein the main component is the disaccharide unit L-iduronosyl-2-O-sulphate-N-sulpho-D-glucosamine-6-O-sulphate”	04-01-2005
	EP0048231	Phadia AB/ Kabi AB	10-09-1981 (15-09-1980 SE)	“Compound/ Oligosaccharide comprising 4-8 monosaccharide units”	29-06-2005
Enoxaparin	US4990502	Sanofi-Aventis	15-04-1988 (16-04-1987 FR)	“Product / A composition of heparins of low molecular weight and their pharmaceutically acceptable salts”	15-04-2008
	US4841041	Sanofi/ Akzo Nobel	12-07-1988 (20-07-1987 EP)	Product/ Pentasaccharide of the formula:	12-07-2008
	EP0287477	Sanofi-Aventis	15-04-1988 (16-04-1987 FR)	“Product / Composition of heparins of which 90% have a molecular weight between 3,600 and 11,000 Da”	15-04-2008
	US5707973	Aventis/Rhone Poulenc	09-12-1994 (23-04-1991 FR)	“Product/mixture of sulfated oligosaccharides having the structure of constituent oligosaccharides of heparin, that has a mean molecular mass of 6±0.6 kD”	13-01-2015
	US5389618 (Re-issued 38743)	Aventis/ Rhone Poulenc	16-07-1993 (26-06-1990 FR)	“Compound/heterogeneous intimate admixture of sulfated heparinic polysaccharides”	14-02-2012
	US5849721	Aventis Pharma SA	06-06-1995 (07-02-1992 FR)	“Product / A mixture of sulphated oligosaccharides obtained from native or depolymerized heparin comprising at least 70% of oligosaccharides having a molecular weight between 5,400 and 7,800 daltons”	15-12-2015

Table 2 (continued)

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
	US6534481	Sanofi-Syhelabo/Akzo Nobel	08-07-1997 (19-07-1996 FR)	“Product/Polysaccharide comprising a region for binding to antithrombin III consisting of a sequence of five monosaccharides bearing in total two carboxylic acid functions”	08-07-2017
	US6617316	Aventis Pharma SA	20-10-2000 (22-10-1999 FR)	64118920891500“Compound/A purified oligosaccharide of formula”;	14-02-2022
	US6969705	Aventis Pharma SA	23-07-2001 (21-07-2000 FR)	“Product/ A composition comprising at least one salt chosen from alkali and alkaline-earth metal salts of at least one sulphated polysaccharide of heparin”	24-10-2021
	US6608042	Aventis Pharma SA	26-03-2001 (28-03-2000 FR)	70231030099000“Product/pharmaceutical composition comprising one or more oligosaccharides of formula”;	26-03-2021
	US7956046	Aventis Pharma SA	27-07-2004 (24-07-2003 FR)	“Compound/An oligosaccharide mixture, wherein: the oligosaccharide mixture comprises an anti-Xa activity of from 200 IU/mg to 450 IU/mg”	22-03-2026
	US7812007	Aventis Pharma SA	01-04-2005 (21-07-200 FR)	“Method/Method of treating the proliferation of smooth muscle cells in a patient”	23-07-2021
	US7687274	Aventis Pharma SA	14-01-2008 (22-02-2004 FR)	“Method/Method of assaying a sample chosen from heparin, low-molecular-weight heparin, ultra low molecular weight heparin, and oligosaccharides”	25-03-2024
	US8492352	Sanofi-Aventis	24-08-2009 (26-08-2008)	“Product/ Synthetic polysaccharide of formula: Methyl (2-[N-(6-aminohexanoyl)-2-deoxy-3,4-di-O-methyl-6-O-sulfonato- α -D-glucopyranosyl)-(1 \rightarrow 4)-(2,3-di-O-methyl- β -D-glucopyranosyl)uronic acid)-(1 \rightarrow 4)-(2,3,6-tri-O-sulfonato- α -D-glucopyranosyl)-(1 \rightarrow 4)-(2,3-di-O-methyl- α -L-idopyranosyluronic acid)-(1 \rightarrow 4)-2,3,6-tri-O-sulfonato- α -D-glucopyranoside sodium salt”	01-09-2029

Table 2 (continued)

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
Tinzaparin B-eliminative cleavage by heparinase/enzymatic depolymerization	US8071570	Aventis Pharma SA	09-05-2011 (10-10-2002 FR)	"Product/ A mixture of sulfated oligosaccharides"	08-10-2023
	EP0244235	Novo Nordisk AS	29-04-1987 (30-04-1986 DK)	"Process/ process for the production of low molecular weight heparin (LMW-heparin) by enzymatic depolymerization of heparin"	29-04-2007
	US5106734	LEO/ Pharmion/Novo Nordisk AS	29-04-1987 (30-04-1986 DK)	"Process/ A continuous process for the production of a low molecular weight heparin product of a predetermined molecular weight by enzymatic depolymerization of heparin"	21-04-2009
Nadroparin Fraxiparin Nitrous acid depolymerization	DE2944792	Choay SA/ GlaxoSmithkline/Aspen	11-06-1979 (11-06-1978 FR)	"Compound/Mucopolysaccharide fraction obtainable from a material based on heparin"	11-07-1999
	US4401662	Choay SA	06-10-1980 (05-10-1979 GB)	"Compound/Oligosaccharide fraction (1) comprises not more than 8 saccharide units, (2) of which one is an N-sulfate-3-O-sulfate-D-glucosamine unit"	30-08-2000
	US4486420	Choay SA	14-09-1981 (06-11-1978 FR)	"Compound/Heparinic mucopolysaccharide fractions"	04-12-2001
	EP0037319	DROPIC/Choay	20-03-1981 (20-03-1981 FR)	"Process/ A process for the preparation of mucopolysaccharides having higher antithrombic activity and a lower ratio of YW/USP"	20-03-2001
	US4500519	Choay SA	20-11-1981 (06-11-1978 FR)	"Process/ A process for making mucopolysaccharide heparinic fractions which have the L-iduronosyl-2-O-sulfate-(1-alpha-4)-N-sulfo-D-glucosamine-6-O-sulfate disaccharide structural units of heparin with the O-sulfated iduronic component of heparin"	19-02-2002
	US4607025	Choay SA	20-12-1982 (28-04-1981 FR)	167894016700500"Compound/A disaccharide of the formula wherein M is hydrogen, --SO ₃ Na or acetyl; Z is --SO ₃ Na or acetyl and R ₁ is hydrogen, methyl or sodium."	19-08-2003

Table 2 (continued)

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
	US4474770	Choay SA	22-08-1983 (05-10-1979 GB)	"Compound/Oligosaccharide fraction (1) comprises not more than 8 saccharide units, (2) of which one is an N-sulfate-D-glucosamine unit"	02-10-2001
	US4686288	DROPIC/Choay	13-12-1984 (20-03-1980 FR)	"Process/ A process for the preparation of mucopolysaccharides having higher antithrombotic activity and a lower ratio of YW/USP and lower YW titer than the starting mucopolysaccharides"	11-08-2004
	US4692435 A	Sanofi-Aventis/ Choay SA	24-03-1985 (06-11-1978 FR)	"Process/ process for obtaining heparinic mucopolysaccharides"	24-12-2004
	US4801583	Choay SA	15-05-1985 (15-01-1982 FR)	10293351016000 "A pure, synthetic tetrasaccharide having the formula."	31-01-2006
	US4804652	Choay SA	19-02-1985 (06-11-1978 FR)	"Process/A process for making mucopolysaccharide heparinic fractions"	14-02-2006
	US4788307	Choay SA and Sanofi-Aventis	29-01-1987 (30-04-1986 FR)	"Compound/An oligosaccharide fraction of the heparin chain which has antithrombotic activity in vivo (as measured by the (Yin-Wessler test) lower than that of heparin"	29-11-2005
	US5599801	Choay SA and Sanofi-Aventis	06-05-1994 (07-05-1993 FR)	"Compound/ heparin fraction resulting from nitrous depolymerization of heparin of natural origin having a content of total N-nitroso compounds not exceeding 500 ppb"	06-05-2014
	EP2314632	Aventis Pharma SA	22-03-2005 (24-03-2004EP)	14136392148100 "Compound/Substantially pure compound having the formula."	22-03-2025
Ardeparin	US4281108	Hepar Industries	02-06-1980 (28-01-1980 US)	"Process/ Process for obtaining low molecular weight heparins endowed with elevated pharmacological properties"	28-01-2000
	US4757057	Pfizer Health AB	07-01-1986 (09-08-1977 IT)	"Process/Method of increasing the antithrombotic activity of mammalian blood relative to the anticoagulant activity"	12-07-2010
	US9068957	Momenta Pharmaceuticals	21-02-2012 (21-02-2011 US)	"Method/Method of evaluating heparin preparations...."	15-08-2032

Table 2 (continued)

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
Sevuparin	WO2009007224A1	Sigma-Tau Industrie Farmaceutiche Riunite S.p.A	10-07-2007 (10-07-2007 EP)	11690355270500 "Compound/A heparin derivative having the formula":	N/A
	US9475888	Dilafor AB	19-12-2012 (19-12-2011 SE)	"Compound/ chemically modified glycosaminoglycan, which glycosaminoglycan is selected from the group consisting of heparin and heparan sulfate"	19-12-2032
	US9480701	Modus Therapeutics AB/ Dilaforette AB	19-12-2012 (19-12-2011 SE)	"Compound/ Chemically modified heparin having an antifactor IIa activity and an antifactor Xa activity"	19-12-2032
	US9480702	Modus Therapeutics AB/ Dilaforette AB	19-12-2012 (19-12-2011 SE)	"Method/A method of treating sickle cell disease"	19-12-2032
	US10023659	Dilafor AB	19-06-2014 (19-06-2013 GB)	"Process/Process for the preparation of a heparin derivative, the process comprising the consecutive steps of: (i) oxidising an acidic aqueous solution of unfractionated heparin by addition of an oxidising agent"	10-09-2034

Table 2 (continued)

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
Parnaparin	US4791195	Opocrin SpA	21-10-1986 (08-03-1983 IT)	“Compound/heparin fraction which is a mixture of oligosaccharides containing an average of 13-17 monosaccharides”	13-12-2005
	US4973580	Opocrin SpA	10-05-1989 (17-05-1985 IT)	“Compound/ The oligosaccharide fraction from dermatan sulfate which has molecular weight 4800 daltons”	27-11-2007
	US5010063	Alfa Wassermann SpA	26-05-1989 (10-06-1988 IT)	“Compound/ Heparin derivative which exhibits signals in the ¹³ C-NMR spectrum at about 53 and about 54 ppm”	26-05-2009
	EP0347588	Alfa Wassermann SpA	19-05-1989 (10-06-1988 IT)	“Compound/Heparin derivative characterized by signals in the ¹³ C-NMR spectrum at 53 and 54 ppm”	19-05-2009
	US5104860	Alfa Wassermann SpA	09-01-1990 (30-01-1989 IT)	“Compound/Heparin derivative having a ¹³ C-NMR spectrum in the zone between 102 and 92 p.p.m. with the presence of a characteristic signal at about 101.3 p.p.m.”	09-01-2010
Reviparin	EP0380943	Alfa Wassermann SpA	12-01-1990 (30-01-1989 IT)	“Process/ process for the preparation of new heparinic derivatives”	12-01-2010
	EP0497162	Alfa Wassermann SpA	17-01-1992 (30-01-1991 IT)	“Process/ Process for preparing Pharmaceutical compositions containing orally absorbable glycosaminoglycans”	17-01-2012
	US5430132	Alfa Wassermann SpA	13-04-1993 (17-04-1992 IT)	“Compound/Glycosaminoglycan of molecular weight 3,000-50,000 Daltons”	13-04-2013
	US5430133	Alfa Wassermann SpA	13-04-1993 (17-04-1992 IT)	“Compound/Glycosaminoglycan of molecular weight 3,000-50,000”	13-04-2013
	US5410039	Alfa Wassermann SpA	07-03-1994 (29-03-1993 IT)	“Process/Product for the synthesis of a product glycosaminoglycan”	07-03-2014
	EP1284717	Abbott GmbH	29-05-2001 (30-05-2000 DE)	“Product/Formulation based on at least one heparin, glycosaminoglycan or heparinoids”	27-12-2006

Table 2 (continued)

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
Bemiparin	EP0293539 US981955	Laboratorios Farmaceuticos Rovi S.A. Lopez Lorenzo L	22-07-1987 (05-01-1987 ES) 26-02-1990 (28-06-1988 US)	“Process/Process of depolymerising heparine having an average molecular weight between 10,000 and 20,000 dalton to produce a depolymerized product” “Process/Method of depolymerizing heparin comprising contacting a fully salified quaternary ammonium salt of heparin with a quaternary ammonium hydroxide in a non-aqueous polar solvent”	22-07-2007 26-02-2010
	EP2308497	Laboratorios Farmaceuticos Rovi S.A.	30-06-2009 (07-01-2008 ES)	“Product/Pharmaceutical composition comprising low or very low molecular weight heparins for use in the treatment of chronic ulcers, characterized in that the plasma half-life of the heparin is between 2.3 and 6.9 hours and the average daily dose of the heparin is between 5,400 and 10,000 IU/day”	30-06-2029
	EP2391352	Laboratorios Farmaceuticos Rovi S.A.	29-01-2010 (30-01-2009 EP)	“A pharmaceutical form comprising a glycosaminoglycan selected from the group consisting of: bemiparin, fondaparinux and enoxaparin and its pharmaceutically acceptable salts”	29-01-2030
	US8802156	Laboratorios Farmaceuticos Rovi S.A.	29-07-2011 (14-11-2007 EP)	“Product/Pharmaceutical form comprising a glycosaminoglycan with antons and a compound presenting cations of pH independent quaternary ammonium groups”	02-04-2029
Certoparin	US4351938	Riker Laboratories	19-05-1980 (19-05-1980 US)	“Process/Process which comprises reacting a heparin salt with from about 5 to 100 milliliters of an aqueous nitrous acid solution per gram of the heparin salt”	19-05-2000

Table 2 (continued)

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
Fondaparinux	US7468358	ParinGenix/Cantex	27-10-2004 (16-06-2004 US)	“Method/Method for treating heparin-induced thrombocytopenia syndrome in a patient comprising administering sulfated polysaccharide comprises 2-O desulfated heparin or 2-O, 3-O desulfated heparin”	07-09-2025
	US8288515	Reliable Biopharmaceutical LLC.	30-07-2010 (31-07-2009 US)	“Compound/A compound of Formula I.”	11-02-2031
	US8420790	Reliable Biopharmaceutical LLC.	29-10-2010 (30-10-2009 US)	“Process/Process for the preparation of a protected heparinic pentasaccharide precursor to Fondaparinux sodium having the structure”	18-04-2031
	US9089484	Novo Nordisk AS	28-03-2011 (26-03-2010US)	“Method/A method for obtaining a reproducible bioavailability of fondaparinux”	13-07-2032
	EP2809678	Reliable Biopharmaceutical LLC	02-02-2012 (02-02-2011 PCT)	“Process/Process for the preparation of a protected heparinic pentasaccharide precursor to Fondaparinux sodium”	02-02-2032
	US9255119	Reliable Biopharmaceutical LLC	18-04-2014 (31-07-2009 US)	“Process/A process for making a compound of Formula I.”	30-07-2030
	CN109734757	Huaibei Normal University	11-03-2019 (11-03-2018 CN)	“Preparation method of fondaparinux sodium injection”	11-03-2039
	CN105175460	Chongqing University	08-09-2015 (08-09-2014 CN)	“Preparation method for monosaccharide fragment intermediate of fondaparinux sodium as anticoagulant drug”	08-09-2035

Table 2 (continued)

LMWH	Patent	Current Owner/ Assignee	Filed on	Claim Type/Claim 1	Patent Expiry
Semuloparin AVE5026	EP1307491 (~WO 02008295)	Aventis Pharma SA	18-07-2001 (21-07-2000 FR)	“Product/Mixtures of sulphated polysaccharides possessing the general structure of the constituent polysaccharides of heparin”	18-07-2021
	EP1556414 (~WO2004033503)	Aventis Pharma SA	08-10-2003 (10-10-2002 FR)	“Product/Sulfated oligosaccharides having the general structure of the constituent polysaccharides of heparin”	08-10-2023
	EP1651677	Aventis Pharma SA	22-07-2004 (24-07-2003 EP)	“Product/Oligosaccharide mixtures having the general structure of the constituent polysaccharides of heparin”	22-07-2024
	US8003623	Aventis Pharma SA	02-08-2007 (10-10-2002 FR)	“Product/Mixture of sulfated oligosaccharides”	19-08-2024
	TW201038279 (~WO2010106519)	Sanofi-Aventis	18-03-2010 (19-03-2009 EP)	“Selection/A dose of 10 mg of AVE5026 for use in therapy in patients with severe renal impairment”	18-03-2030 (If granted)
	WO2012072799A1	Aventis Pharma SA	02-12-2011 (02-12-2010 EP)	“Assay/A method for the in vitro measurement of the biological activity of an Ultra Low Molecular Weight Heparin (ULMWH) sample, wherein said method is carried out relative to a standard comprising an ULMWH (semuloparin).”	N/A
	US9346894	Sanofi-Aventis	11-04-2012 (11-04-2011 FR)	“A sulfonated polysaccharide having a polysaccharide of heparin which has a molecular weight of less than 8000 Daltons”	23-09-2032
RO-14 (a derivative of Bemiparin)	EP1070503	Laboratorios Farmaceuticos Rovi S.A.	13-10-1999 (23-07-1999 ES)	“Product/Composition of heparin of very low molecular weight, with the general formula:”	13-10-2019
	US6384021	Laboratorios Farmaceuticos Rovi S.A.	03-11-1999 (23-07-1999 ES)	“Product/Composition of heparin of very low molecular weight, with the general formula:”	03-11-2019
	EP2881404	Laboratorios Farmaceuticos Rovi S.A.	02-08-2013 (02-08-2012 ES)	“Process/ Process for obtaining very low molecular weight heparin (VLMWH)”	02-08-2033

Source: <http://www.patents.google.com>; <http://www.epo.org>; <http://www.wipo.org>; <http://www.uspto.com>; <http://www.drugfuture.com>

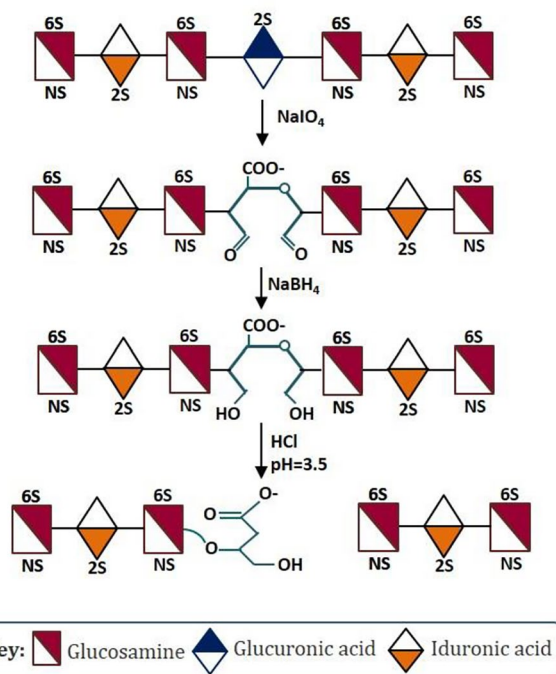
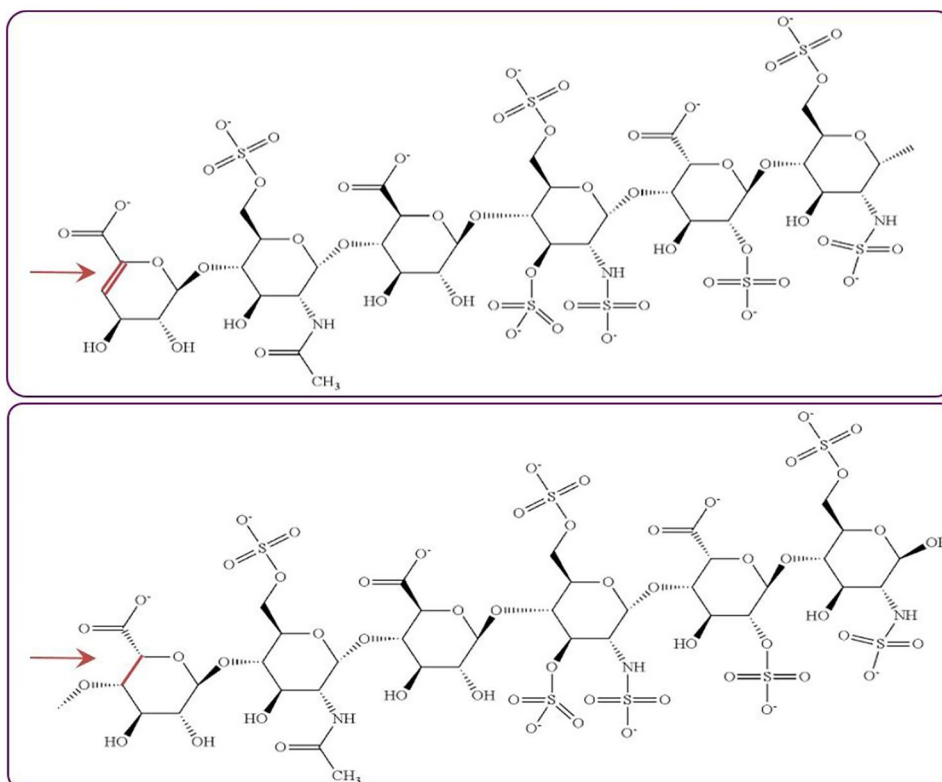


Fig. 3 Schematic reaction scheme of sevuparin sodium preparation

on the presence of two ATIII-binding sites in high molecular weight heparin fractions of 18,000 to 22,000. In parallel, analysis of LMWHs having MW ~ 6000–8000 Da had

Fig. 4 Polysaccharides comprising two antithrombin III-binding hexasaccharide sequences. When hexasaccharide is located at the NRE of the polysaccharide, the bond between carbon atoms 4 and 5 of the first saccharide is a double bond; else, the bond between carbon 4 and 5 of the first saccharide is a single bond (Source: Mourier P. and Viskov C. Polysaccharides comprising two antithrombin III-binding sites, preparation thereof and use thereof as antithrombotic medicaments. US9346894B2 (2016))



established the presence of single site for interaction with Viskov et al.'s very hypothesis that LMWH can interact with at least two AT proteins and consequently to be having at least two possibilities of inhibiting factor Xa has unveiled a new generation of hemisynthetic LMWHs (~ 2000 to 3000 Da) with a new antithrombotic profile [30].

Additionally, current approaches to develop selective factor Xa inhibitors for oral administration such as synthetic pentasaccharides (fondaparinux) in the form of water-in-oil microemulsion (Novo Nordisk), rivaroxaban (Bayer Health-Care AG and Scios, Inc.), apixaban (Bristol-Myers Squibb), and 813,893 (GlaxoSmithKline) are promising and provide a reproducible and predictable bioavailability of the LMWHs (see US9346894B2).

Continuous development of new heparin fragments with distinct characteristics and clinical applications and, in parallel, gradual expiry of old patents and data exclusivity are clear indicators of an ever competitive and a never-ending market for heparin and heparin-derived products worldwide. Controlling quality pre- and post marketing is the core issue that need to be addressed squarely especially in countries where local pharmaceutical industry is lacking modern laboratory facilities at one hand, while compromising good manufacturing practices at the other. In the next section, the article lays out how reorientation in analytical approaches can better characterize LMWH structure and compositions and discover new applications of known heparin and heparin-derived products.

Advanced Analytical Approaches for LMWH Characterization and Control of Quality in Patent Ecosystem

Since the first generic LMWH enoxaparin entry into the US market in 2010, quality assurance and reproducibility with claimed safety and efficacy have become even greater challenges for FDA and other regulatory authorities worldwide. The very first inquiry for assessing generic/biosimilar drug product similarity with the innovator drug product is what scientific data are required to sanction a conclusion that the generic/biosimilar drug product has the same active ingredient as the innovator drug product for which quality and safety stand established by the innovator company. While clinical studies can support a conclusion of generic/biosimilar drug product safety and effectiveness, these studies are inert towards distinguishing the two drug products at the molecular levels [3]. Recognizing the scientific and regulatory complexities in the way to approval for generic LMWH drug products, and 2007–2008 heparin contamination crises, FDA presented a framework that requires characterization of several key aspects of LMWH drug products with advanced analytical techniques in the active ingredient sameness assessment quest. The framework is followed by many developing countries that are members of WTO's agreement on TRIPs *mutatis mutandis* while many least developed countries have adopted the FDA framework as *verbatim* transcription.

Conventional approaches for structural characterization of LMWHs (such as proton and carbon nuclear magnetic resonance (NMR) spectroscopy, capillary electrophoresis (CE) and mass spectrometry (MS)) have unveiled many of their critical quality attributes; nonetheless, the chemical diversity of GAG polysaccharides resulting from a variety of differential pattern of O- and N-sulfation and uronic acid C-5 epimerization creates a need for development of new approaches for understanding the still latent structural and functional attributes of LMWHs as a measure to control quality and improving their clinical applications [33]. To support the inference that modes of depolymerization used to produce generic and innovator LMWH drug products produce the same degree and cleavage pattern of parent heparin, FDA has made determination of MW distribution and relative abundance of oligosaccharides of different MWs, analyzing the overall chemical composition in the generic LMWH drug product sourced from two different manufacturers and contrasting them with the innovator LMWH drug product a mandatory requirement. This adds to scoring the need for still more analytically sensitive, specific, and technically impeccable approaches for profiling LMWH complex and heterogeneous mixtures with great variations in sulfation and sequence composition [28]. In this quest, advances

in (i) mass spectrometry (such as LC–MS, CE-MS, CE-LIF (laser-induced fluorescence) and tandem mass spectrometry (also known as MS/MS or MS²)) that involve pairing of two or more mass analyzers for increasing their capacity to analyze; (ii) online separations including HPLC (such as size-exclusion chromatography (SEC), strong anion exchange (SAX), reverse-phase ion pairing (RPIP), and hydrophilic interaction chromatography (HILIC)), ion mobility spectrometry (IMS), high-field asymmetric waveform ion mobility spectrometry (FAIMS), and capillary zone electrophoresis (CZE); and (iii) automated analysis software have greatly revolutionized the top-down and bottom-up approaches for precise structural characterization of LMWHs [8]. Table 3 provides a listing of modern analytical tools for LMWH fine structural and compositional analyses that also need listing in the US and EU pharmacopoeias for assuring quality and purity of the LMWH drugs approved for marketing.

As an outcome of these innovative efforts, today, researchers and pharmaceutical industry have in their toolkits several independent and hyphenated approaches for LMWHs' deeper structural and compositional analyses including but not limited to the following: (i) 2D-NMR for identifying LMWH types and calculating their monosaccharide composition (1D ¹H-NMR identification test is part of US pharmacopoeia and EU pharmacopoeia monographs for heparin or LMWH sodium) [34]; (ii) 2D ¹H-¹³C- heteronuclear single quantum coherence (HSQC) spectroscopy for characterizing normal structural variations in the intact LMWH molecular composition [34] (Using the approach, commercially available LMWHs (enoxaparin, dalteparin and tinzaparin) were tested for results. The technique identified the major as well as the minor components present at the termini of the oligosaccharide chains.) [35]; (iii) 2D-chromatography combining SEC and RPIP approaches, offering better resolution of LMWH components of different sizes and of same sizes but with different charges and polarities, and providing information on both size distribution and sequences of LMWHs for control of quality (Using electrospray quadrupole time-of-flight (Q-TOF) mass-spectrometer more than 80 oligosaccharides in nadroparin and more than 120 in enoxaparin were identified.) [36]; (iv) HILIC-LC–MS for characterizing major chains of LMWH octadecasaccharide in size [37]; (v) ultra-performance size exclusion chromatography (UPSEC) coupled to electrospray quadrupole time-of-flight (Q-TOF) mass-spectrometer (UPSEC-Q-TOF–MS) [38] for profiling LMWH oligosaccharide chains with sizes upto dp30 (The technique identified more than 70 components in enoxaparin including oligosaccharides with unnatural structures 1,6-anhydro rings and saturated uronic acid

Table 3 Patent position and pros and cons of analytical tools that generics can utilize for profiling generic/biosimilar LMWH drug products

Technique	Advantages	Challenges	Patent	Inventor(s)	Current assignee	Patent expiry
RP-LC-MS	Capability to perform intact chain mapping analysis; offer rich information about chain distribution and composition of LMWHs; can identify trace amounts of unusual structures or impurities, can characterize by-products, and degradation products	Insufficient separation resolution, detection sensitivity or specificity; restricted ability to identify different disaccharide building blocks of LMWHs (12 to 17)	US7329353	Thomas Dillon et al.	Amgen Inc	06-03-2026
CE-MS	Capability to perform intact chain mapping analysis; offer rich information about chain distribution and composition of LMWHs; can identify trace amounts of unusual structures or impurities rapid, economic, ultra-sensitive	Insufficient separation resolution, detection sensitivity or specificity; restricted ability to identify different disaccharide building blocks of LMWHs (12 to 17)	WO2015121366	Johann FAR et al.	Analisis S.A	N/A
LIT-MS/MS/MS (or MS ³)	Better in-trap fragmentation, high ion trap sensitivity, and high selectivity	Difficult high selection resolution	US9852895	Daniel James Kenny	Micromass UK Ltd	20-01-2030
MSI-CE-MS	High-throughput screening of ions within a single capillary; enhanced data processing with quality assurance	Low mechanical robustness, poor reproducibility	US9490110	Phillip Britz-McKibbin	McMaster University	14-05-2034
CE-ESI-MS	Simple, inexpensive, good in resolution, easy capillary replacement, highly compatible with MS	Insufficient capacity, low selectivity, not suitable for high MW analysis	EP2250490B1	Elizabeth Jane Maxwell, Xuefei Zhong, Hong Zhang, David Da Yong Chen	University of British Columbia	06-03-2029
ESI FT-ICR MS	Ultra high spectral resolution and mass accuracy; facilitate application of top-down MS strategies	High cost, requires large space, long signal acquisition time, not suitable for clinical laboratory applications	US8530834	Alan G. Marshall et al.	Florida State University Research Foundation, Inc	05-02-2030
DE-MALDI-TOF MS	High spectral resolution and mass accuracy, obviating the need for MS tuning during single sample signal acquisition	Presents different results under similar sample preparation using similar conditions	US9536726B2	James VanGordon, Bradford Clay	Biomerieux, Inc	27-08-2035

Table 3 (continued)

Technique	Advantages	Challenges	Patent	Inventor(s)	Current assignee	Patent expiry
SEC-UPLC/Q-TOF HRMS	Simple; robust; capacity to reflect process-based structural changes; potential to reveal contribution of each individual components to the overall bioactivity of LMWHs; capacity to identify oligosaccharide with sizes upto dp30. * [37]	Cost, insufficient storage capacity	US202002000719A1	Navya Sama et al.	Alembic Pharmaceuticals Ltd	Pending (05–09-2038, if granted)
IP-HILIC LC-ESI-MS	Simple, effective for analysis of –vely charged saccharides, suitable for coupling with MS, low IP concentration increases resolution and strength of MS, highly sensitive, low contamination rate, useful for quality control of LMWHs	Sensitivity fluctuates during runs, low separation efficiency for polar compounds	WO2016051170A1	Lingzhi Gong	Isis Innovation Limited	N/A
HILIC-UPLC-CAD/MS	Capacity to separate highly sulfated and very polar heparin oligosaccharides; universal adaptability to any MS instrument; capacity to identify oligosaccharides with sizes up to dp28; robust, reproducible and easy-to-use, offers impressive increase in sensitivity when compared with RP-LC-MS	High reliance on aprotic solvent acetonitrile, less flexible	US9441053B2	ChungYao Wang, Imin Huang, Chia Yen Wu, YungTe Chiang Helen Chao	ScinoPharm Taiwan Ltd	20–02-2035
SEC-MS/RPIP-HPLC-MS	Fast, capacity to disclose size distributions and sequences; better in oligosaccharide resolution, provide interesting information about oligosaccharide structure, chain length, and chemical modifications	Expensive, low tolerance to contaminants	WO2013139478A1	Giangiacomo Torri, Antonella Bisio	Istituto di Ricerche Chimiche e Biochimiche “G. Ronzoni”	N/A

Table 3 (continued)

Technique	Advantages	Challenges	Patent	Inventor(s)	Current assignee	Patent expiry
SAX-HPLC or RP-HPLC ESI-MS	Capacity to profile disaccharide building blocks; offers better resolution; signal enhancement, effective in costs	Signal suppression, low tolerance to contaminants	US9139876	Zachary Shriver, Naveen Bhatnagar, Nur Sibel Gunay, Jennifer Ozug, Elaine Y. Sun	Momenta Pharmaceuticals, Inc	23-08-2031
Orbitrap MS/MS	High resolution/ionization efficiency; high mass accuracy; miniature design, offers identification and characterization of all known disaccharide building blocks	Lower sensitivity, false negatives, low-abundance peaks	US7728290B2	Alexander Makarov	Thermo Finnigan LLC	22-10-2024
HPLC-FTICR + LTQ-FTMS	Posttranslational modification analysis of complex molecules	Low-abundance peaks, limited mass accuracy and resolution	WO2006026569A2	Shiaw-Lin Wu et al.	Northeastern University	N/A
LC-MS/MS	Capable of sequencing short oligosaccharide mixture with saccharides as large as dodecasaccharides; highly sensitive, specific and rapid in detection	Higher operational cost; more limited sample throughput; less favorable concentration sensitivity	US8945933	Anthony Le, Tina Cowan	The Board of Trustees of the Leland Stanford Junior University	20-10-2032
FT-ICR MS	Incomparable high mass resolution, mass measurement accuracy; small, simple	Relatively slow acquisition rate	US5886346	Alexander Alekseevich Makarov	Thermo Finnigan LLC	29-03-2016
IMS-MS	Simple, selective, sensitive, inexpensive,	Low-resolution, limited dynamic response range, low tolerance to contamination	US9607820B2	Robert Harold Bateman et al	Micromass UK Ltd	21-05-2030
ECD-CID MS/MS	Simple, low costs, selectively cleaves c-z site on the amino acid backbone; suitable for analyzing post-translational modifications	Low in fragmentation efficiency; requires high signal-to-noise ratio for precursor ions	US8080786B2	Takashi Baba, Hiroyuki Satake, Izumi Waki	Hitachi High Tech Corp	10-07-2026

Table 3 (continued)

Technique	Advantages	Challenges	Patent	Inventor(s)	Current assignee	Patent expiry
CID MS/MS	Creates MS spectra with high accuracy and efficiency; produce useful fragmentation	Reduces multiplicity of bond cleavages; Ineffective for large molecule samples; Easily detached functional groups cause loss of fragment-derived information on location and provide limited structural information	US8269166	Daisuke Okumura	Shimadzu Corp	05-02-2029
IRMPD MS/MS	Fast, free from blind spots, no degradation of high vacuum; allows product ion formation on axis	Fails to conduct complete structural analysis; easily loses side chains involves in post translational modifications of biomolecules; allows secondary fragmentation	US6717137B2	Steven A. Hofstadler, Jared J. Drader	Ibis Biosciences Inc	11-06-2022
ETD MS/MS	Cleaves main amino acid residues sequence chain while preserving the post-translationally modified site; allows more complete sequence information; works well with partially ionized precursor ion; can be practiced on a variety of other analytical tools	Not feasible for large-scale analysis, high in cost; technically complex	US8692187	Donald F. Hunt et al.	University of Virginia Patent Foundation	19-04-2025
ECD MS/MS	Efficient, suitable for analyzing complex mixtures and large biomolecules, produce more structurally important cleavages than CID and IRMPD; produce simple/predictable fragmentation pattern	Can be employed in Penning cell ion cyclotron resonance mass spectrometers	US6958472	Roman Zabarev	Syddansk Universitet	22-03-2022

Table 3 (continued)

Technique	Advantages	Challenges	Patent	Inventor(s)	Current assignee	Patent expiry
EID MS/MS	Suitable for dissociating high-mass, even-electron ions produced by thermo spray and other soft ionization tools; yields extensive and reproducible fragmentation characteristics; can analyze qualitatively and quantitatively greater quantity of samples; can be easily implemented on a variety of analytical tools	Need electron sourcing and singly charged precursor ions	US4731533	Marvin L. Vestal	Applied Biosystems LLC	15–10–2006
IT-MS	Selectively trap multivalent ions having variety of charge to mass ratio	Can cause outgassing, Need for large radio frequency (RF) potentials can aggravate RF breakdown	US6847037	Yoshikatsu Umemura	Shimadzu Corp	19–05–2023
2D-Tandem MS	Much faster, offers large increase in peak capacity, can characterize intact chains or originally stable non-fragmenting ions	High cost	US6770871	Houle Wang, Kerry D. Nugent	Bruker Corp Bruker Scientific LLC	01–06–2022
Chip cube Nano-LC-ESI-MS/MS ion-trap system	Easy, reliable, efficient, highly sensitive, can investigate a wide range of biomolecules	Reproducibility, sensitivity dependency on shape of the capillary tip and cone distance	US20160305919A1	Gregory Staples	Agilent Technologies Inc	Abandoned

RP-LC-MS reverse phase-liquid chromatography-mass spectrometry, *CE* capillary electrophoresis, *LIT* linear ion trap, *FT-MS* Fourier transform mass spectrometry, *FTICR* Fourier transform ion cyclotron resonance, *MSI* multi-segment injection, *DE* delayed extraction, *MALDI* matrix-assisted laser desorption ionization, *ESI* electrospray ionization, *IT* ion trap, *CID* collision-induced dissociation, *IRMPD* infrared multiphoton dissociation, *HPLC* high-performance liquid chromatography, *HILIC* hydrophilic interaction chromatography, *Q-TOF* quadrupole time-of-flight, *RPIP* reversed phase ion pairing chromatography, *SEC* size exclusion chromatography, *UPSEC* ultra-performance size exclusion chromatography, *SAX* strong anion exchange chromatography, *CTA-SAX* cetyltrimethylammonium-coated SAX, *UPLC* ultra-performance liquid chromatography, *HRMS* high-resolution-MS, *UVPD* ultra-violet photodissociation, *EDD* electron detachment dissociation, *EID* electron-induced dissociation, *NETD* negative electron transfer dissociation

Sources: (i) For patents: www.patents.google.com, www.epo.org, www.uspto.com, www.wipo.org; (ii) For advantages and disadvantages of techniques: Lauren E. Pepi, Patience Sanderson, Morgan Stickney, I. Jonathan Amster, Developments in Mass Spectrometry for Glycosaminoglycan Analysis: A Review, *Molecular & Cellular Proteomics*, Volume 20, 2021, 100,025, ISSN 1535-9476, <https://doi.org/10.1074/mcp.R120.002267>; (iii) Zhangjie Wang, Lianli Chi. Recent advances in mass spectrometry analysis of low molecular weight heparins [J]. *Chin. Chem. Lett.*, 2018, 29(1): 11–18

at the non-reducing ends.) [39]; (vi) HILIC LC–MS supported by Agilent’s chip-based nanospray amide HILIC LC–MS system to analyze LMWHs up to dp18 [40]; (vii) a universal diol-based HILIC Fourier transform (FT)-ESI–MS platform for direct characterization of intact LMWHs (coupled with bioinformatics software package (GlycReSoft 1.0) [41], a quantitative comparison of up to 200–500 components in two commercial LMWH products, innovator enoxaparin (Lovenox) and generic enoxaparin, was made possible.) [42]; (viii) LC–MS for profiling LMWH longer chains (More than 80 compositions were identified using the method.) [35]; (ix) RPIP-ESI–MS for profiling intact LMWHs (Using the method, Chi et al. identified more than 200 intact components in enoxaparin sodium.) [43]; (x) a HILIC-MS for separating highly sulfated and polar heparin oligosaccharides using ambient mobile phases with low ammonium salt concentration [44]; (xi) CE-MS for intact chain mapping analysis with speed and cost-effectiveness [45]; (xii) RPIP-ESI–MS method performed on an ion trap-time-of-flight hybrid mass spectrometer IT-TOF for oligosaccharide fragments mapping [46]; (xiii) HILIC, multiple reaction monitoring (MRM)-MS methods for identifying and quantifying disaccharide building blocks [47] (HILIC-MRM-MS has been used successfully for dalteparin and nadroparin disaccharide building blocks analysis.) [48]; (xiv) LC-MRM-MS for determining composition of disaccharide building blocks; (xv) ion mobility spectrometry (IMS) — a powerful technique for GAGs separation based on ions mobility, when coupled to MS/MS resolves GAG negative ions having the same mass-to-charge ratio [49] and analyze GAG chains qualitatively and quantitatively [33]; (xvi) electron-based ion activation techniques (such as collision-induced dissociation (CID), infrared multi-photon dissociation (IRMPD), electron-induced dissociation (EID), electron detachment dissociation (EDD), ultra-violet photodissociation (UVPD), and negative electron transfer dissociation (NETD) for extensive crossing cleavages of polysaccharide chains), allowing identification of epimers, distinction between IdoA and GlcA, number of sulfo-modifications, determination of position of sulfo-group substitutions [50], and sequence information of highly sulfated GAGs [51]; (xvii) gated trapped IMS paired with NETD MS/MS for characterization and quantification of highly sulfated GAG isomers without decomposition of sulfo-group [52]; (xviii) online LC and CID MS/MS for sequencing chemically derivatized mixtures of oligosaccharides [53]; (xix) CZE coupled with NETD MS/MS for structural analysis of GAG in human urinary samples [54]; (xx) HILIC-LC–MS for analyzing GAG oligosaccharides up to dp30 [55] (Applying the technique to a mixture of dalteparin and nadroparin,

depolymerized through nitrous acid, Sun et al. separated and identified 36 building blocks) [56]; (xx) HILIC LC-NETD MS/MS for separating and sequencing chemically synthesized tetra- and hexasaccharide isomers without permethylation [57]; and (xxi) traveling wave ion mobility spectrometry (TWIMS)-MS for separating and characterizing mixtures of chondroitin sulfate oligomers [58].

Evaluating the strengths and efficiencies of analytical advances made in the past decade, the CE-based method for separating and determining the ratio of 1,6-anhydro structure in enoxaparin as developed by Kang and Zhan and disclosed in US 9012229B2 is a substantial advancement in the fine structural analysis of enoxaparin sodium [59] and for controlling the drug product quality during manufacturing. The method could separate and measure, qualitatively and quantitatively, all the building blocks of enoxaparin including disaccharides, trisaccharides, tetrasaccharides, and specifically oligosaccharides having 1,6-anhydro ring structure. The US Pharmacopoeia (USP) has used ratio of 1,6-anhydro as a standard in drug product quality control of enoxaparin. In an exhaustively digested product of enoxaparin sodium with a mixture of heparin lyases I, II, and III in a ratio of 1:1:1, Kang and Zhan detected the following components: (i) four oligosaccharides with 1,6-anhydro ring structure; (ii) a trisaccharide of structure I; (iii) 8 disaccharides represented by the symbols — Δ IA, Δ IS, Δ IIA, Δ IIS, Δ IIIA, Δ IIIS, Δ IVA, and Δ IVS; (iv) two non-naturally occurring disaccharide Δ IIS_{gal} and Δ IVS_{gal} comprising a galacturonic acid produced by 2-O-desulfation of -IdoA(2S)-GlcNS(6S) and -IdoA(2S)-GlcNS; (v) two 3-O-sulfo containing tetrasaccharides (Δ IIA-IISglu and Δ IIa-IVSglu) [60] may or may not be having affinity to ATIII.

The structural identification of above components received endorsement from the USP monograph for enoxaparin sodium [61]. For calculating the molar %age of oligosaccharide chains with 1,6-anhydro ring structure in enoxaparin sodium, Kang and Zhan used the following equations/formulae:

$$1, \text{ 6Anhydro \%} = W_x \times \left(\frac{w_{16+17}\%}{443} + \frac{w_{14}\%}{545} + \frac{w_{15}\%}{1210} \right)$$

$$1, \text{ 6Anhydro \%} = 100 \times W_x \times \frac{(Area_{14} + Area_{15} + Area_{16} + Area_{17})}{\Sigma(Mw_x \times Area_x)}$$

Wherein W_x is the weight-average molecular weight of enoxaparin sodium.

The W_x of enoxaparin sodium was 4500 Da (US5389618A) ranging from 3800 to 5000 Da wherein (i) about 20% oligosaccharides have MW less than 2000 Da, (ii) more than 68% oligosaccharides have MW between 2000 and 8000 Da, and (iii) no more than 18% oligosaccharides

have MW higher than 8000 Da (US9012229B2). The characteristic oligosaccharide chains in enoxaparin contain a pentasaccharide sequence, originally displayed in the parent heparin polysaccharide chains, that accounts for 15–25% in enoxaparin sodium.

Adding to the LMWH fine structural characterization efforts as one of several measures to control quality of pharmaceutical drug product during first manufacturing as a whole and subsequently in batch-to-batch variation, in 2017, Arnold et al. reported on the development of a new technique that they claimed to be more reliable, sustainable, and efficient for determination of M_w and MW distribution of LMWHs [18]. The research team coupled chemo-enzymatically synthesized oligosaccharides with a predictive *in silico* model, developed from a library of chemosynthetically synthesized heparin oligosaccharides for enoxaparin MW determination. The group believed that the chemo-enzymatic process allows production of pure oligosaccharides that is reproducible and can be used as standard for a MW analysis of enoxaparin. To have their approach translated into practice, Arnold group had accomplished the following: (i) synthesized a panel of oligosaccharides covering the enoxaparin MW range between 2226 and 5176 Da; (ii) developed a predictive *in silico* model for MW analysis; (iii) developed a set of guidelines for testing system suitability; and (iv) analyzed commercially available enoxaparin from different manufacturers [18]. While Arnold group acknowledged that using refractive index (RI) detection method in lieu of HPLC as stated in the USP monograph is a limitation of the approach, nonetheless development of homogenous oligosaccharide for MW analysis of LMWHs is promising and must be projected as a measure to ensure quality and consistency of the product [18]. In the same year, Bisio et al. [14] worked on a similar approach of combining different analytical strategies (such as LC–MS and NMR) for deep structural characterization of LMWHs particularly dalteparin. The group found the approach effective for comparative studies of dalteparin samples or assessment of batch-to-batch variability [14].

For addressing the pharmaceutical quality control issue, Karawdeniya et al. [62] reported the use of silicon nitride nanopores for easy differentiation of clinical heparin sample and contaminated over-sulfated chondroitin sulfate (OSCS). In another study for LMWH control of quality, Im et al. [63] used solid-state nanopores sensor with a support vector machine (SVM) learning algorithm for GAGs single molecule identification and quantification. The technique is capable of identifying impurity in a heparin sample with high accuracy (> 90%) at the level of 0.8% (w/w). Still, another study implicating production and characterization of LMWHs for better therapeutic results and providing an alternative anticoagulant therapy is made by Oliveira et al. [64] Working on the proposed strategy, the group first produced and characterized LMWH nanoparticles through the

solvent-evaporated double emulsion method, making use of poly(lactic-co-glycolic acid) (PLGA) and poly(vinyl alcohol) (PVA), followed by quantification of encapsulation efficiency (EE), and evaluation of stability. Homogenous and stable nanoparticles with low polydispersity index (IPD) of 0.067 ± 0.05 and EE value of 66.5% suggested effectiveness of the nano-encapsulation method and its use as an alternative anticoagulation therapy. [64]

The research group — Stickney et al. [65] — has made parallel attempts for structural characterization of LMWHs in 2019, making use of LC–MS, UHPLC–MS, CE–MS, and MS/MS techniques. Recently, Miller et al. [66] used a combinatorial approach coupling NETD and CE and applied it to LMWH (enoxaparin) deeper characterization. The strategy allowed enoxaparin separation within 30 min, identified 37 unique molecular compositions, and assigned 9 structures using MS/MS [66]. In another latest study, Saad and Leary [67] developed a “shotgun ion mobility mass spectrometry sequencing” (SIMMS²) for heparin saccharide intact chain mapping. Contrasting data for intact and fragment ions against standards HS oligosaccharide structure has allowed determination of heparin unnatural saccharides including 3-O-sulfo groups containing variants [67].

Bioinformatics Tools for LMWH Oligosaccharide Mass Spectra Annotation to Succeed Control over Drug Product Quality

Apart from advances in analytical approaches for detailed structural characterization and compositional analysis of LMWHs, either as part of regulatory compliance for marketing approval or pharmacovigilance after the drug products have been licensed for use, efficient software/bioinformatics tools that allow accurate analysis of datasets or interpretation of spectra generated by various analytical techniques (such as LC/MS, LC–MS/MS) are equally desirable to achieve the drug product quality control and consistent reproduction goal. In the past one decade, several bioinformatics tools have been developed for translating and/or interpreting mass spectra into meaningful information. Starting from early developed software package HOST for heparin/HS oligosaccharide sequencing [68], today, researchers and pharmaceutical industry have variety of choices for undertaking identification of GAG oligosaccharides different structures and elemental composition analysis. Amongst these, bioinformatics tools include the following: (i) GlycoWorkbench [69] for analyzing carbohydrate mass spectra through matching an artificially generated library of possible glycan structures, fragments, and compositions using all known and modified disaccharide building blocks against the dataset of peaks derived from the MS sequencing (the system aims to fully

facilitate the everyday manual interpretation of MS data); (ii) Manatee for rapid extracting, assigning, and contrasting glycan compositions from LC–MS datasets [70]; (iii) GlycReSoft [71] for processing bottom-up and top-down data auto-processed by open source software Decon2LS/DeconTools to generate the identity and quantitative information for LMWHs; (iv) GlycYouCan by Kinoshita et al. to unify databases for extensive glycan analysis [72]; (v) GlycCompSoft for automated comparison of LMWHs using top-down LC/MS data [73]; (vi) GlycoDeNovo — an efficient algorithm for accurate de novo glycan topology reconstruction from tandem mass spectra [74]; (vii) GAGfinder — the first software package for MS/MS spectrum peak ending and elemental composition analysis of GAG [75] (GAGfinder is a targeted approach to spectrum analysis and annotating peak isotopes composition that exploits precursor product information for generating hypothetical library of fragments for experimental result matching); and (viii) GAG-ID — a multivariate mixture model to estimate the automate assignment of LC–MS/MS-fragmented derivatized heparins [76].

In addition to getting benefits from the above analytical advances for commercial LMWH quality and consistent reproduction, many pharmaceutical companies are outsourcing characterization of unfractionated heparin/LMWH samples to the commercial companies. Such companies may provide drug manufacturers a broad range of structural and physicochemical data sufficient to sustain rigorous assessment of LMWH similarity with the originator drug product by the regulatory authorities [77].

Analytical advances for structural characterization of uniquely modified and more complicated LMWHs than the precursor heparin are increasingly helping the new and generic LMWH drug product manufacturers in the successful preparation of their application dossier for marketing authorization approval process at one end while the regulatory authorities (FDA-EMA-DRAP-Health Canada etc.) to revise and/or upgrade heparin sodium monographs in light of these advances to ensure release of quality and safe generic/biosimilar LMWH drug products in the local and global markets.

Current Pharmacopeial Monographs for LMWH Marketing Approval

While new advances in technology influence compendial standards for drug products characterization and quality assurances as a matter of routine, adverse drug reactions or immunogenic responses may also require development of new tests and specifications not only to address the concerns but also to improve the quality and purity of the drug product in question. Following 2007–2008 heparin contamination crisis with over-sulfated chondroitin sulfate (OSCS),

the pharmacopoeias for LMWHs had undergone extensive revisions worldwide. In the USA, FDA urged the need for introduction of new analytical approaches for detecting the presence of OSCS impurities in the heparin preparations and tools for determination of MW distribution and weight-average Mw in the heparin monograph of the USP as a measure to control quality and purity [78]. Succeeding 2009 revision in enoxaparin sodium monograph, new assays including 1D ¹H NMR, CE, and SAX-HPLC have been added in the USP for detection of impurities in the heparin sodium monograph [79]. While different regulatory authorities have set the level of impurity tolerance at varying degree, the USP for heparin sodium and heparin calcium has set specification for galactosamine-containing glycosaminoglycans component not more than 1% [19].

For MW determination, Mulloy et al. [80] reported on the development of a broad standard calibrant for USP heparin sodium MW calibrant reference standard (RS). The group also developed a simplified SEC method with light scattering detection for determination of MW distribution of heparin sodium for incorporation into the USP to help ensuring safety and quality of heparin sodium [80]. Following experts' discussions and lapse of public comment period, the below criteria was accepted for incorporation into the USP heparin sodium monograph [81]:

- i) $M_{24,000}$ not more than 20%
- ii) M_w between 15,000 and 19,000
- iii) The ratio of $M_{8000-16000}$ to $M_{16,000-24,000}$ not less than 1.0

This development has made direct comparison between MW values for standard heparin determined by different laboratories possible [80].

Another effort for MW determination using homogenous calibrants as a measure to control quality testing has been reported by Arnold et al. [18] in 2017. The group used support vector machine technique for modeling as a reliable, consistent, and improved substitute for USP recommended nonlinear regression analysis to account for enoxaparin's heterogeneity. The previously used standards (i.e., mixtures of oligosaccharide derived from the heparin source material) because of heparin source heterogeneity have imprecise structure, hence difficult reproduction.

In countries like Australia, Canada, and Pakistan, the respective regulatory authorities (Therapeutic Goods Administration (TGA), Health Canada, and DRAP) have adopted *mutatis mutandis* principal revisions in USP for enoxaparin sodium while European Medicines Agency (EMA) has defined their own reference standards for assessment of enoxaparin's quality, purity, and efficacy. Generally, EMA follows EP Reference Standards H0185000 for heparin low molecular mass for assay BRP (biological reference preparations) and Y0001282 for

heparin physico-chemical analysis but, specifically, from quality perspective, EMA requires performance of extensive comparability studies encompassing the following: (i) comparison of heparin source material and mode of depolymerization; (ii) physicochemical characteristics; (iii) structural comparison for assessing oligosaccharide sequence similarity, oligosaccharide fragment similarity, disaccharide building block similarity, and high affinity/no-affinity component similarity; (iv) in vitro biological assays (clotting tests such as activated partial thromboplastin time [aPTT] and HEPTEST) and biochemical activity (inhibition of coagulation factors Xa (anti-FXa) and factor IIa (anti-FIIa) [82]. Apart, EMA also requires the following: assessment of degree of sulfation and sodium content of the oligosaccharide chains, MW determination using HP-SEC with Triple Detector Arrays, and assessment of monosaccharides forming an integral part of the disaccharide building blocks in enoxaparin chain including assessment of residues at both the reducing and non-reducing ends. From clinical perspective, EMA can accept a single PK/PD study or otherwise drop a dedicated efficacy trial provided a comparison of the physicochemical characteristics, biological activity/potency, and PD fingerprint profiles of a candidate biosimilar and the reference product leads to a convincing inference of “similar efficacy,” based on the use of highly sensitive and specific methods [83].

In Australia, TGA mostly follows the Ph. Eur. monographs for LMWHs (0828) [84] (EUP-LMWH-2014) and enoxaparin sodium (1097) [85], but additional considerations are also given to the following: mass-average relative molecular mass percentage, benzyl alcohol, nitrogen, loss on drying, and anti-factor Xa and anti-factor IIa assays [86].

In Pakistan, the DRAP follows the US Pharmacopoeia and British Pharmacopoeia standards for testing the quality and comparability of LMWH biosimilars with the reference listed drug. In particular, DRAP requires the following: The weight average molecular weight of LMWHs must be less than 8000 Da; more than 60% of the total must have MW less than 8000 Da; and anti-Fxa/anti-IIa ratio must be greater than 1.5 [87]. The DRAP (Registration Board) has declared that drug products registered in any of the regulatory authorities, namely US FDA, EMA, PMDA Japan, TGA Australia, and Health Canada shall receive registration grant. In addition, the molecules/formulations (in same dosage form) and clinical trials approved by the regulatory authorities of UK, Germany, France, Switzerland, Netherlands, Austria, Denmark, Sweden, and Norway will also be taken as reference/guidelines for consideration of Registration Board, as authorities of these countries have robust drug regulatory mechanisms and long standing strong litigation systems. However, the DRAP (Registration Board) reminded the stakeholders that should the domestic circumstances be

justifying, they shall also consider their own safety and efficacy parameters of the drug; and decision shall be made on case-by-case basis.

Regulatory Pathways for Generic/Biosimilar Marketing Approval: FDA Exempts ANDA Applicants for Generic LMWHs to Submit Clinical Studies While EMA, WHO, Health Canada, TGA, and DRAP Sanction the Requirement

In USA, ANDA pathway specified under Sect. 505(j) of Federal Food, Drug, and Cosmetic Act of 1938 (FDCA) is exclusive for generics (small molecule drugs) approval while Biosimilar Biologics License Applications (BBLA) pathway specified under 351(k) of Public Service Health Act (PHSA) is reserved for biosimilars. FDA considers unfractionated heparin and LMWHs a drug (not a biologic), hence requires applicants to follow NDA pathway as specified under Sect. 505(b)(1) and 505(b)(2) of FDCA for new LMWHs and ANDA pathway (505(j)) for generic LMWH approval [88]. Placing reliance on the FDA's previous findings about the clinical safety and effectiveness of originator drug product, an ANDA applicant is not required to submit clinical studies for re-establishing safety and efficacy of the generic drug product. In contrast, EMA, WHO, Health Canada, and DRAP consider LMWHs as biologically active products and hence urge the need for conducting clinical trials to reliably predict pharmacological effect of the biosimilar LMWHs.

Generally, a generic drug product approved following ANDA pathway is considered to be therapeutically equivalent to the reference drug product in terms of safety and clinical efficacy and hence can be substituted at the pharmacy-level without additional formalities (such as physician recommendation, dose adjustment, safety monitoring) [3]. For generic LMWH drug products, however, since a different source material and mode of depolymerization (chemical or enzymatic) introduces unique changes in the structure and consequently pharmacological activity of the resulting LMWHs, FDA considers each NDA LMWH to be a different drug product and hence not substitutable at the pharmacy level [89]. US FDA approved the first generic version of enoxaparin in 2010 after satisfying itself that the generic enoxaparin contains a 1,6-anhydro ring structure at the reducing ends of between 15 and 25% of its oligosaccharide chain. This new elemental approach for generic enoxaparin approval was the consequence of Aventis's citizen petition No. FDA-2003-P-0273 that required FDA to withhold approval of any generic version of enoxaparin unless the generic manufacturer satisfied certain conditions including the

presence of signature structure (1,6 anhydro) at the reducing ends of the polysaccharide chains. Recognizing the scientific and regulatory complexities in the way to approval for ANDAs for enoxaparin, FDA developed a five-tier approach each of which assesses the active ingredient ‘similarity’ goal from a different perspective. Summarily, the five-tier approach includes assessment of equivalence/biosimilarity between the generic enoxaparin and the reference drug product (Lovenox®) in respect of the below aspects: (i) physicochemical attributes; (ii) heparin source material and mode of depolymerization; (iii) nature and arrangements of components that constitute enoxaparin (such as disaccharides building blocks, fragment mapping, and sequence of oligosaccharides species); (iv) laboratory measurements of anticoagulant activity (biological and biochemical assays for determining anti-factor Xa and anti-factor IIa activity); and (v) drug effects on humans (in vivo pharmacodynamic profile) [89].

The equivalence evaluation against the five-tier criteria is based on a comparative qualitative and quantitative analysis of generic enoxaparin for the multiple batches of the originator enoxaparin. Such comparability exercise takes into account the expected batch-to-batch variability, sampling, and analytical test variability expected from every biological product. Equivalent molecular diversity tilts the balance of probabilities towards equivalence in the clinical efficacy and safety profiles of the generic and the originator enoxaparin. Depending upon the future advances in the analytical techniques used for characterization of LMWHs, FDA may require a different or improved criteria, or tests for active ingredient similarity demonstration and quality assurance [28].

In European Union, EMA has introduced a centralized procedure under Article 3(2)(b) of the Regulation (EC) No. 726/2004 for generic enoxaparin sodium marketing approval. This procedure meets EMA’s Committee for Medicinal Products for Human use (CHMP) agreement. Apart from complete quality data, EMA/CHMP requires enoxaparin biosimilar manufacturers to support application dossier for a similar biological medicinal product with non-clinical and clinical data [82]. However, in cases where similar efficacy can be strongly inferred from a comparison of the physicochemical attributes, biological activity/potency, and PD fingerprint profiles, EMA may withdraw clinical efficacy studies. According to EMA, enoxaparin biosimilarity assessment exercise is not a “one-stop service.” A technically sound combination of conventional and modern techniques and optimization of Ph. Eur. based parameters and tests are desirable for full characterization of LMWHs as part of biosimilarity assessment exercise. EMA based their marketing authorization decisions on “totality-of-evidence” approach and clinical data derived from comparative PK/PD trial establishing biosimilarity at the level of quality, clinical safety, and efficacy.

In Australia, all marketing authorization applications are considered and published by TGA. For biosimilar version of enoxaparin, TGA follows the US FDA multiple point criteria for approval [28]. For comparability exercise, TGA follows the EMA guidelines on biosimilar drug products [90]. In parallel, TGA gives due consideration to the conclusions drawn by the reference member states (such as New Zealand, Mexico, and EU) on biosimilar enoxaparin approval. Following EMA’s *Guideline on non-clinical and clinical development of similar biological medicinal products containing low-molecular-weight-heparins using complex mixtures*, TGA may allow clinical efficacy studies where the generic and reference drug products sponsors have established convincing similarity in the analytical characteristics (structural and functional attributes) and PD fingerprint profiles [91].

Parallel to the position in Australia and Health Canada, DRAP (Pakistan) in its 281st meeting held on April 11–13, 2018, adopted the same 5-tier approach as developed by US FDA when they approved enoxaparin ANDA pathway in 2010 [3]. In particular, following FDA guideline, DRAP requires applicants seeking approval of biosimilar version of enoxaparin (Lovenox) to provide the multiple point data/information along with application Form-5A under Rule 26(1) of the Drugs (Licensing, Registering and Advertising) Rules, 1976, for registration of an imported enoxaparin drug. Prior to reaching their conclusion, DRAP had taken into account WHO Technical Report Series No. 999 (2016), wherein the Health Canada pointed out the oversight in the regulatory approval pathway for LMWHs and clarified that these should be regulated as biologicals and not as small-molecule drugs [92]. Subject to (i) establishing convincing similarity/equivalence to the innovator drug product as per the USP monograph at the levels of physicochemical properties; active pharmaceutical ingredient sameness; levels of impurities, weight-average Mw, and distribution of sequences of disaccharide building block units in the oligosaccharide chains; and in vitro biological and biochemical assay results; and in vivo PD profile and (ii) assurance by the manufacturer that a confirmatory clinical efficacy and safety trial would not provide any additional information about the drug product safety and efficacy than previously established by the innovator company, DRAP (Registration Board) may waive a pre-approval phase III efficacy/safety trial to avoid duplicating efforts and resources.

Conclusion

While development of new strategies for structural characterization of highly complex LMWHs and discovering their new clinical applications is a continuing challenge, so far, advances in analytical techniques (such as “in silico” modeling, digitalization, orthogonal separations and online

testing) are encouraging for LMWH control of quality and purity during manufacturing and post-marketing. A continuum of patented or non-patented high-throughput separation techniques coupled to high-resolution mass spectrometry (MS, MS/MS) and bioinformatics platforms for spectral interpretation have made GAG chains analysis fast, more accurate, dependable, and meaningful for scientists and laboratory researchers. Where these scientific advancements and increased technical know-how are resulting in development of new therapeutic drug targets, understanding the process-dependent variations in structural characteristics of LMWHs, and discovery of new applications of old active ingredients, in appropriate cases (where a sponsor of generic drug product provides a reasonable scientific justification for the same), these may let regulatory authorities to relax or obviate the need for expensive and extensive in vivo animal testing and clinical trials [92]. Foregoing clinical trials subject to rigorous scientific evidence in support of active ingredient sameness quest may increase availability of low-cost generic/biosimilar LMWHs to the public at one hand, save substantial cost for the health care system at the other, a patent-friendly ecosystem where pharmaceutical and biotechnology companies may have increasing opportunities to make substantial investments in innovation and development. In parallel, advances in uses of old structural analysis tools such as CE and NMR are providing new tracks for innovative thinking and translating them into practice.

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Data Availability All data analyzed/literature reviewed is included in the article.

Declarations

Conflict of Interest The authors declare no competing interests.

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