Production and Chemical Processing of Low Molecular Weight Heparins

ROBERT J. LINHARDT, PH.D. AND NUR SIBEL GUNAY, M.S.

ABSTRACT **Heparin is an animal tissue extract that is widely used as an anticoagulant drug. A number of low molecular weight heparins (LMWHs), introduced in the past decade, are beginning to displace pharmaceutical (or compendial) grade heparins as clinical antithrombotic agents. This article describes the chemical properties of the glycosaminoglycan (GAG) heparin and how it is prepared and processed into pharmaceutical grade heparin. There are several commercially produced LMWHs that are prepared through the controlled depolymerization of pharmaceutical grade heparin. The chemistry of the commercial processes used for manufacturing LMWHs is discussed. Structural differences are found in the LMWHs prepared using different commercial processes. Careful control of process variables has generally resulted in the reproducible preparation of LMWHs that are structurally uniform and of high quality. The specifications, however, remain different for each LMWH. Thus, LMWHs are a group of similar but different drug agents. As the structural properties of LMWHs vary significantly, the bio-equivalence or inequivalence of these agents must ultimately be established by the pharmacologists and the clinicians.**

Keywords: **Low molecular weight heparin, analysis, structure, process, production**

Heparin, a clinical anticoagulant, has been one of the most effective and widely used drugs of this century.[1,2] As one of the oldest drugs currently still in widespread clinical use, heparin is unique as it is among the first biopolymeric drugs and one of only a few carbohydrate drugs. Indeed, heparin's introduction predates the establishment of the United States Food and Drug Administration.[2] Low molecular weight heparins (LMWHs), also referred to as low molecular mass heparins (LMMHs), are a group of heparin-derived anticoagulant/antithrombotic agents that began their development during the last quarter of this century.[3–7]

The introduction of LMWHs primarily resulted from an improved understanding of the molecular basis of the biochemistry associated with the coagulation cascade.[8–10] The isolation of the serine protease inhibitor, antithrombin III (AT), and the characterization of coagulation factors (serine proteases), such as thrombin and factor Xa (inhibited by AT), were critical in driving the development of LMWHs.[7,8] Heparin accelerates the inhibition of these coagulation factors by AT, preventing the generation of a fibrin clot. In the coagulation cascade, one factor activates the next until prothrombin (factor II) is converted to thrombin (factor IIa) by factor Xa. It is thrombin that acts on fibrinogen to form a fibrin clot.[8] The very nature of this cascade suggested a therapeutic opportunity to develop an agent that was more specific than heparin (which acts at many points in the cascade) and that might provide more subtle regulation of coagulation and reduce the major hemorrhagic side effects associated with heparin. LMWHs were originally developed based on this rationale. Various laboratories observed that when heparin is fractionated based on size or broken down chemically or enzymatically, its activity against thrombin is decreased to a much greater extent than its activity against factor Xa.[11–15] The separation of activities result from differences in their molecular requirements for inhibition. Factor Xa interacts directly with AT bound to a specific pentasaccharide sequence in heparin (the AT binding site) and requires a short heparin chain comprised only of these saccharide units for its inactivation.[5] In contrast, thrombin must also bind adjacent to AT on a flanking sequence in heparin, thus requiring a longer heparin chain with 18 or more saccharide units for its inactivation.[3,8] As factor Xa lies at the convergence of the extrinsic and intrinsic pathways of the coagulation cascade, it was speculated that a

Department of Medicinal and Natural Products Chemistry, University of Iowa, College of Pharmacy, Iowa City, Iowa.

Reprint requests: Dr. Linhardt, Professor of Pharmacy and Chemical Engineering, University of Iowa, College of Pharmacy, PHAR 303A, University of Iowa, Iowa City, IA 52242.

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LMWH having an enhanced anti-factor Xa/anti-factor IIa ratio would facilitate more subtle regulation of coagulation and an improved therapeutic index.[8–10]

Our understanding of the precise mechanism of action of LMWHs through biochemical, pharmacological, and clinical studies have suggested that the initial rationale for their development may have been naive and not entirely correct.[7,8] Nevertheless, LMWHs have been successfully introduced as new effective and improved antithrombotic/anticoagulant agents throughout the world. This review focuses on the chemical processing and production of LMWHs and how the various processing routes result in structural differences among these pharmaceutical agents.

WHAT IS HEPARIN?

Within a decade of its discovery in 1916, heparin was identified as an anionic polysaccharide containing a uronic acid residue[2] (Fig. 1). Early researchers showed that heparin also contained *O*-sulfate esters and *N*-sulfated glucosamine residues. By 1970, iduronic acid was shown to be the major uronic acid component in heparin and a generalized structure of heparin could be drawn. Over the past two decades, the structure of the AT pentasaccharide binding site has been discovered with much of heparin's fine structure elucidated, and an improved understanding of its conformation[21–23] and interaction with proteins was established.[16–18,20,24–27]

Heparin is prepared by extraction from the tissue of slaughter house animals (i.e., porcine intestine, bovine lung). Like all other natural polysaccharides, heparin is a polydisperse mixture containing a large number of chains having different molecular weights (MWs).[28,29] Heparin is composed of a major trisulfated disaccharide repeating unit (Fig. 1), but it also contains a number of additional disaccharide structures.[18–20] It is these additional disaccharide units that make heparin's structure complex and that also comprise the AT pentasaccharide binding site, important for heparin's anticoagulant activity.

The heparin family of glycosaminoglycans (GAGs) includes both heparin and the related undersulfated polysaccharide heparan sulfate.[30,31] While heparin and heparan sulfate GAGs are biosynthesized through a common pathway, structural studies clearly indicate that the structures of heparin and heparan sulfate are distinctly different.[32,33] All the disaccharides found within heparin, including those comprising the AT pentasaccharide binding site[34], are also found within heparan sulfate but in different proportions.[30] Heparin and heparan sulfate, both found in tissues commonly used to prepare pharmaceutical grade heparin, differ substantially in their anticoagulant activity.[30] Extraction methods that focus on the high specific anticoagulant activity required to meet United States Pharmacopeia (USP) specifications serve to eliminate much (but not all) of the heparan sulfate GAG from pharmaceutical grade heparin.

Pharmaceutical grade heparin is a purified tissue extract comprised primarily of polydisperse GAGs consisting primarily of heparin but containing other GAGs, such as heparan sulfate. Small amounts of dermatan sulfate, once present in some pharmaceutical grade heparins, have now been virtually eliminated.[18,28,35] Chains of molecular weight from 5000 to over 40,000, making up polydisperse pharmaceutical grade heparin, also display significant sequence heterogeneity.[1] For example, many fully sulfated heparin chains are simply composed of uniform repeating sequences of trisulfated disaccharide (Fig. 1). Alternatively, heparin chains having an intermediate level of sulfation are comprised of long segments of fully sulfated sequences with intervening undersulfated domains, such as that comprising the AT pentasaccharide binding site (Fig. 1). Finally, undersulfated heparin chains (<2 sulfate groups/disaccharide) may simply be contaminating heparan sulfate.

Not all heparin chains contain an AT pentasaccharide binding site. Only 20 to 50% of the polysaccharide chains comprising pharmaceutical grade heparin contain an AT binding site and are called "high affinity heparin."[1] No difference has been reported in the overall charge or the average size of high affinity and low affin-

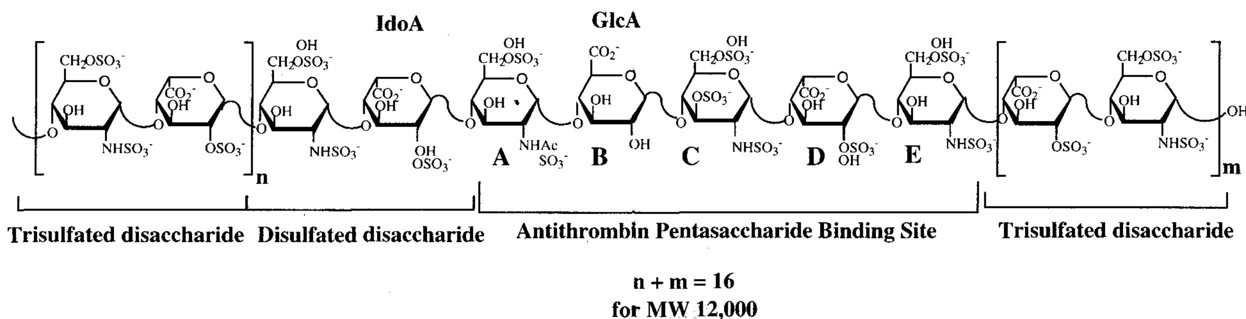

FIG. 1. Chemical structure of a representative chain of pharmaceutical heparin. Clusters of trisulfated disaccharides (n and m, where n + m = 16 for MW 12,000), flank disulfated disaccharides and AT pentasaccharide binding site (ABCDE). Some structural variability

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TABLE 1. Composition of Heparins from Different Species and Tissues*

		Average Number in One Heparin Chain†			
Species	*Tissue*	*N-acetyl AT Binding Site*	*N-sulfo AT Binding Site*	*Trisulfated Disaccharide*	*Disulfated Disaccharide*
Porcine	intestine	0.5 (0.3–0.7)	0.1	10 (10–15)	1.2(1–2)
Bovine	lung	0.3	0.3	14	1.0
Bovine	intestine	0.3	0.3	10	1.7
Ovine	intestine	0.7	0.4	11	1.4
Hen	intestine	0.3	0.2	6.7	1.7
Clam	-	0.5	0.4	5.0	1.9

*adapted from Loganathan et al.[19]
†The numbers shown in parentheses indicate a range of values typically observed.

ity heparin. Some high MW heparin chains may contain more than a single AT binding site and thus display an enhanced level of anticoagulant activity.[36]

Heparins obtained from different tissues and different species also differ structurally (Table 1).[19] The most widely used tissue for the preparation of pharmaceutical grade heparin is porcine intestine. Heparin prepared from bovine lung differs substantially from porcine intestinal heparin. Bovine lung heparin has a higher sulfation level and slightly higher MW than porcine intestinal heparin, increasing its affinity for thrombin (factor IIa). Porcine intestinal heparin contains an AT binding site primarily having an *N*-acetyl group in residue A (Fig. 1), while bovine lung heparin primarily has an *N*-sulfo group at residue A, resulting in their slightly different affinities for AT.[19]

The disaccharide composition of porcine intestinal heparins can also differ substantially from each other.[18] The two mainly used raw materials (intestinal mucosa and whole intestine) contain differing amounts of contaminating heparan sulfate that can carry over into the final pharmaceutical product. There are different subspecies of hogs and the mast cell content of intestinal tissue can vary based on the diet and environment in which the animals are raised. These variables potentially contribute to the already complex structure of pharmaceutical grade heparin.

HOW IS HEPARIN PREPARED?

Methods of commercial production of pharmaceutical grade heparin are tightly guarded industrial secrets and few publications or patents describe most commonly used pharmaceutical processes. The process of preparing pharmaceutical grade heparin has been altered somewhat over time as the primary tissue source has changed from dog liver to beef lung and finally to porcine intestine.[37] The methods used today for the commercial preparation of heparin involve five basic steps: (1) preparation of tissue; (2) extraction of heparin from tis-

The preparation of the tissue begins with the collection of the appropriate animal organ tissue at the slaughterhouse and its preparation for processing. The whole intestine is either used to prepare "hashed pork guts" or processed into casings, which requires removal of the endothelial lining from the intestinal lumen. Crude heparin extraction typically takes place at the hog slaughtering facility itself. Additional high potency heparin may be recovered by saving the waste brine solution of the hog casings operation.[38]

In the second step, heparin is separated from the tissue. The use of elevated temperatures and pressures[39] and/or proteases ensures the solubilization of all GAGs. Currently, commercial crude heparin extraction processes involve hydrolysis at alkaline pH aided by proteolytic enzymes. Optionally, the digested tissue may be filtered or screened to remove any large particles yielding a deeply colored solution containing GAGs, peptides, and nucleic acids. At this point, the enzyme is often inactivated by heating the filtrate for 15 minutes at 90°C, at the same time serving as a sanitary step.

The third step is the recovery of raw heparin. Currently, anion exchange resin is added, enabling the heparin-like GAGs to selectively adsorb onto the resin[40] according to the charge-density of the different GAGs. After complete adsorption of the heparin, the resin is delivered to a crude heparin manufacturing facility where it is washed and subsequently eluted. The concentrated crude heparin solution is usually filtered, precipitated, and vacuum dried (stage 12 heparin).

The purification of crude heparin is typically performed under good manufacturing practices conditions and deals with potential impurities originating from the starting material or introduced during crude heparin extraction. Generally, the crude heparin is dissolved in purified water, filtered at low pH to remove residual protein, and oxidized at alkaline pH to sanitize, decolorize, and depyrogenate the material. This is often followed by cation exchange chromatography to remove extraneous cations, ethanol precipitation to reduce nucleotides, and sometimes

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either vacuum-dried or redissolved in purified water, followed by various filtration steps and freeze-drying. The yield of porcine intestinal heparin is typically 10–25 mg/g wet tissue corresponding to 30,000 to 50,000 U/animal.

WHAT ARE LMWHS?

LMWHs are defined as salts of sulfated GAGs having an average MW of less than 8000 Da and for which at least 60% of all molecules have a MW of less than 8000 Da. These are obtained by fractionation or depolymerization of heparin and have a potency of greater than 70 units/mg of anti-factor Xa activity and a ratio of anti-factor Xa activity to anti-factor IIa activity of ≥ 1.5.[41]

Before any LMWHs had been approved for human use, the implicit goal of pharmaceutical scientists was that the composition of these LMWHs should closely resemble the structure of heparin in all aspects except MW and ratio of anti-factor Xa to anti-factor IIa activity. An ideal LMWH might be simply a LMW subfraction of heparin prepared by sizing, using gel permeation chromatography (GPC). Direct size fractionation has been used to prepare a LMWH on a laboratory bench scale that exhibits the appropriate MW and activity properties, and contains the same disaccharide composition and sequences as heparin. Such methods, however, are rarely used on the scale required for the commercial manufacture of heparin.[42] Pharmaceutical chemists have relied on a number of chemical or enzymatic depolymerization methods to manufacture commercial quantities of LMWHs. These depolymerization methods were selected to give a product with: (1) suitable average MW and low polydispersity; (2) anti-factor Xa/anti-factor IIa activity >1; (3) structure similar to a LMWH prepared through fractionation and with few structural artifacts resulting from the depolymerization method used; (4) no residual toxic reagents;[43] and (5) a cost-effective reproducible and scalable process having a minimum number of process steps, little if any required purification, neither labor, reagent nor capital intensive, and high yielding. While no current manufacturing process meets all of these goals, the currently used processes have afforded a first generation of clinically useful LMWHs.

METHODS FOR PREPARING LMWHS

Many years of experience in the manufacture of pharmaceutical grade heparin have shown that it exhibits a surprisingly high level of physical and chemical stability with a shelf life approaching a decade. Numerous processes have been used to prepare pharmaceutical grade heparins involving the use of harsh conditions including elevated temperature, pressure, shear, high ionic strength, acid, base, and organic solvents. These processes have resuccess of the manufacturers represented the only available data demonstrating the physical and chemical stability of heparin. Recently, an accelerated stability study under elevated temperatures and under acidic and basic conditions confirmed the surprising stability of heparin.[44] A decomposition pathway for heparin under these stressed conditions has been proposed. Elevated temperatures can result in substantial damage to functionality within the heparin molecule (i.e., loss of sulfation) that occurs concurrently with depolymerization. Neutral and acidic pathways result in a similar formation of small desulfated products, while the basic pathway terminates in a Cannizzaro reaction and de Bruyn van Eckenstein rearrangement.[42] Other physical parameters such as agitation result in no structural alterations, as the heparin molecule is not sufficiently large to be shear-sensitive.

The oxidative instability of heparin had been widely observed by heparin manufacturers. Indeed, antioxidants (bisulfite and metal chelators) have been added at various stages in heparin's manufacture to enhance stability.[42] These observations suggested the possibility of utilizing oxidative methods to prepare LMWHs. Manufacturers have also observed microbial degradation of heparin. A bacterial enzyme, heparin lyase I (heparinase), is known to act on heparin.[45,46] This enzyme acts in a random endolytic fashion through a β-eliminative cleavage mechanism.[46] This enzymatic reaction can be mimicked chemically by esterifying the carboxyl group of the uronic acid residue and treating the resulting heparin ester with base.[47] Thus, enzymatic or chemical β-eliminative cleavage offers a second possible method for heparin depolymerization and the manufacture of LMWHs. It is interesting to note that many of the processes to prepare LMWHs started out as analytical tools to degrade heparin in an effort to understand its structure.

Heparin can be oxidatively broken down using a variety of oxygen containing reagents like hydrogen peroxide or by ionizing γ-irradiation (Fig. 2).[11,48–50] Each of these methods relies on the generation of oxygen radicals that are believed to act by oxidizing sensitive saccharide residues within the heparin polymer. Nonreducing sugars are essentially inert to aqueous hydrogen peroxide except in the presence of alkali or in the presence of a metal catalyst. Both of these conditions lead to the generation of the hydroxyl radical that will react with sugar residues and degrade them to 1-, 2- and 3-carbon fragments without modifying the residues on either side of the point of attack. The most susceptible residues appear to be those that are unsubstituted at positions 2 and 3 in the sugar ring. Studies of the composition of disaccharides that result from oxidative depolymerization of LMWHs suggest that nonsulfated uronic acid residues in heparin are selectively oxidized to volatile acids (i.e., formic acid).[51] Under controlled conditions (temperature, pressure, time, oxidant), LMWHs having appropri-

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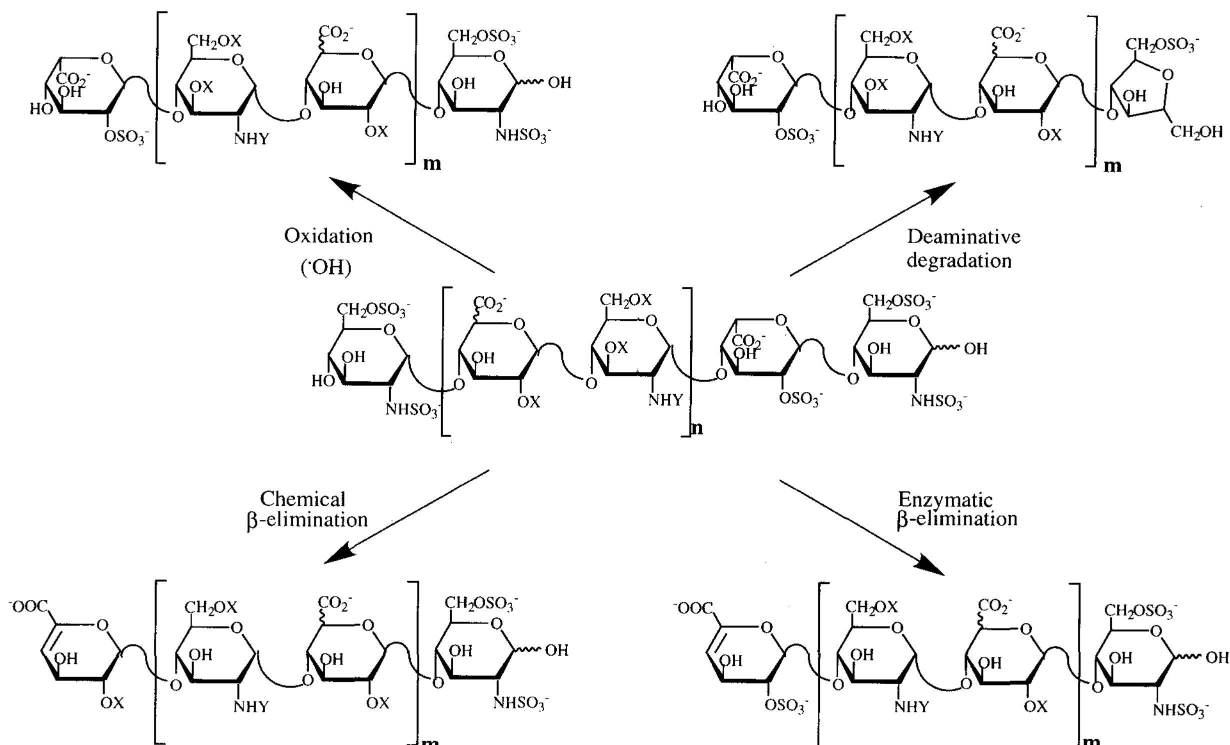

FIG. 2. Depolymerization of heparin to prepare LMWHs. The heparin chain in the center can undergo depolymerization by each of the four processes shown. Heparin chain size is reduced (n > m), affording a LMWH.

yields.[11,48,52] Of these oxidative methods, only hydrogen peroxide has been utilized to commercially prepare LMWHs (ardeparin sodium and parnaparin sodium) for clinical use (Table 2).

In addition to oxygen radical processes, it is possible to oxidatively depolymerize heparin through deamination (Fig. 2). In these reactions, heparin is *N*-nitrosated, using either nitrous acid or another nitrosating reagent such as isoamyl nitrite, at the amino group of its *N*-sulfoglucosamine residues. The resulting unstable *N*-nitrososulfamide loses nitrogen and sulfate and generates a carbocation at C-2 of the saccharide residue. Subsequent ring contraction of this residue and hydrolysis of the adjacent glycosidic bond affords a LMWH. Each product chain resulting from this process contains an anhydromannose residue (bearing a terminal aldehyde) at the reducing terminus. This residue can subsequently be converted to anhydromannitol using a reducing agent, such as sodium borohydride. Controlled deaminative cleavage is possible by controlling the process condi-

TABLE 2. Commercially Available LMWHs

LMWH	*Trade Name*	*Manufacturer*	*Preparation Method*	*Approved Markets*
Ardeparin sodium	Normiflo	Wyeth-Ayerst	Oxidative depolymerization with H_2O_2	USA
Certoparin sodium	Sandoparin	Novartis	Deaminative cleavage with isoamyl nitrite	Germany
Dalteparin sodium	Fragmin	Pharmacia-Upjohn Kissei	Deaminative cleavage with nitrous acid	USA, Japan UK, Germany
Enoxaparin sodium	Lovenox Clexane	Rhône-Poulenc Rorer Avantis	ß-eliminative cleavage of the benzyl ester of heparin by alkaline treatment	USA Germany Spain
Nadroparin calcium	Fraxiparin	Sanofi-Winthrop	Deaminative cleavage with nitrous acid	France Germany
Parnaparin sodium	Fluxum	Alfa Wassermann	Oxidative depolymerization with Cu^+ and H_2O_2	Italy
Reviparin sodium	Clivarin	Knoll	Deaminative cleavage with nitrous acid	Canada Germany
Tinzaparin sodium	Innohep Logiparin	Braun Novo/Leo/Dupont	ß-eliminative cleavage by heparinase	Germany Denmark

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