SEMINARS IN THROMBOSIS AND HEMOSTASIS—VOL. 25, SUPPL. 3, 1999

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.

0094–6176/1999/E1098–9064(1999)25:S3:0005–0016:STH00547X

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-

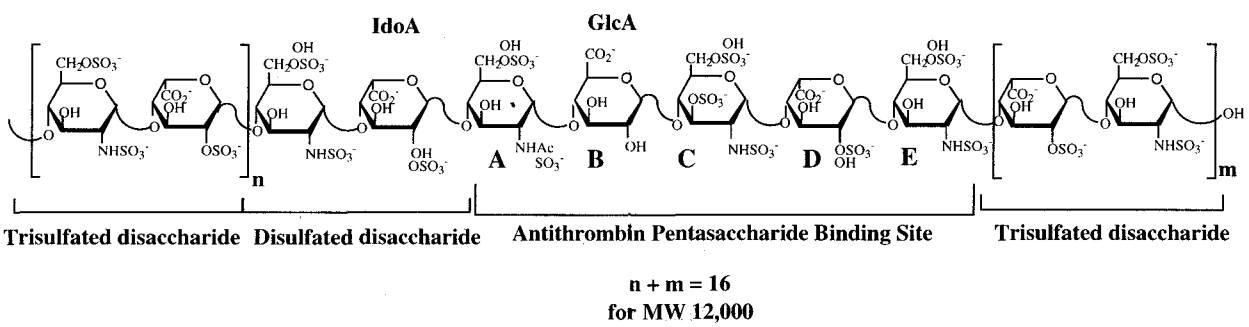

FIG. 1. Chemical structure of a representative chain of pharmaceutical heparin. Clusters of trisulfated disaccharides (n and m, where n + m = 16 for MW 12,000), flank disulfated disaccharides and AT pentasaccharide binding site (ABCDE). Some structural variability both within and outside the AT binding site is indicated by multiple substituents. The attached substituents correspond to the major AT binding site structure found in porcine intestinal heparin.

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 tissue; (3) recovery of raw heparin; (4) purification of heparin; and (5) recovery of purified heparin.

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 by a chemical treatment to inactivate any viruses that might be present. The purified heparin is precipitated and

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 reproducibly afforded a uniformly high quality product. The success 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 appropriate MWs and activity can be obtained in reasonable

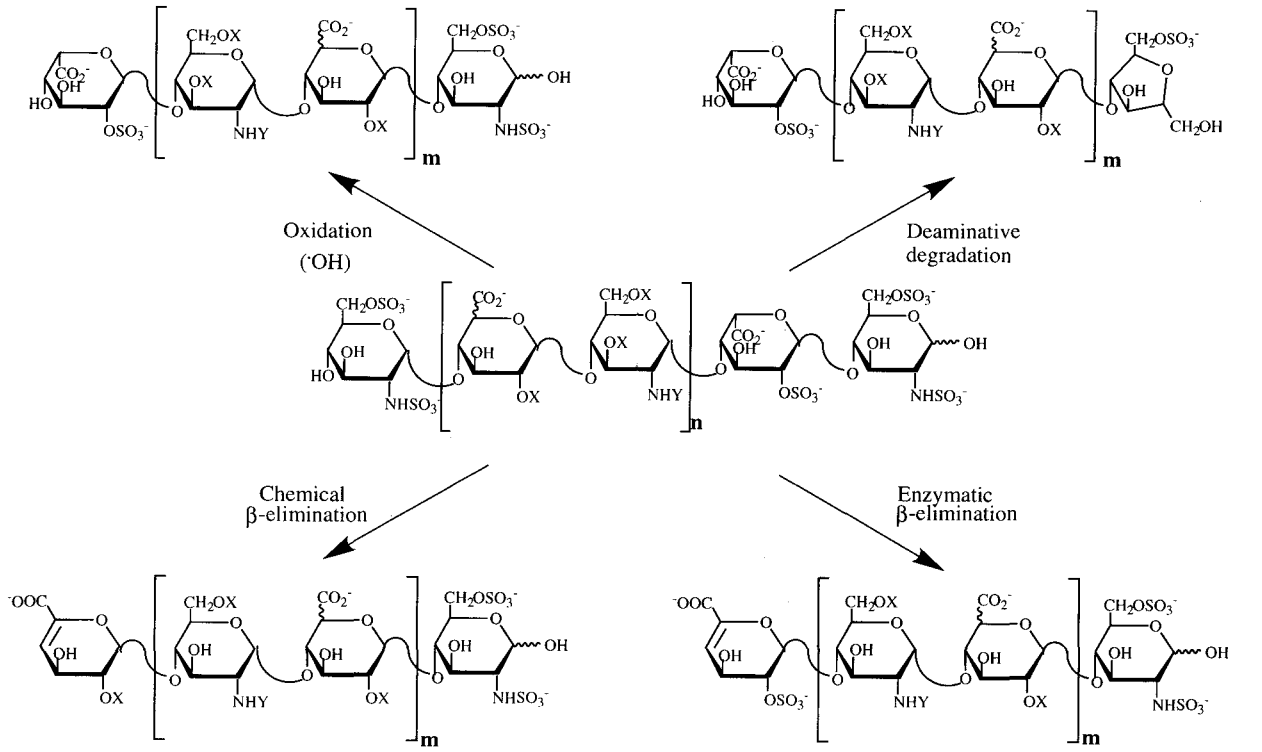

FIG. 2. Depolymerization of heparin to prepare LMWHs. The heparin chain in the center can undergo depolymerization by each of the four processes shown. Heparin chain size is reduced (n > m), affording a LMWH.

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, USA

tions (temperature, pH, time) or by limiting the amount of nitrosation reagent.[53,54] The LMWH product formed using these controlled conditions is obtained in high yield and has the appropriate chemical and biological properties. Several LMWHs prepared through deaminative cleavage are currently used clinically (Table 2).

Two β-eliminative methods, one enzymatic and the other chemical, are used to commercially prepare LMWHs. In the enzymatic method, heparin lyase I (or heparinase) is used to depolymerize heparin.[15] The extent of this reaction can be conveniently monitored by measuring the change in absorbance associated with the unsaturated uronate residue formed in each product molecule.[46,55] Cleavage takes place only at the 2-*O*-sulfoiduronic acid residue.[46] The depolymerization is stopped by removing or inactivating the enzyme. After recovery of the GAG from the enzyme and very low MW byproducts (i.e., disaccharides and tetrasaccharides), a LMWH is obtained that has the desired MW and activity properties. This method is used to prepare the clinically used LMWH product, tinzaparin sodium (Table 2). Chemical β-elimination can involve the direct treatment of heparin or its quaternary ammonium salt with base.[56,57] Alternatively, the benzyl ester can be prepared by treatment of the benzethonium salt of heparin with benzyl chloride and base with heating.[58] Under these conditions, chemical β-elimination takes place, affording LMWH that contains an unsaturated uronate residue in the nonreducing end. Cleavage occurs specifically at iduronic acid without preference for the presence or absence of a 2-*O*-sulfo group. Proper control of these process conditions affords the clinically used LMWH, enoxaparin sodium (Table 2).

ANALYSIS OF HEPARIN AND LMWHS

The analysis of pharmaceutical grade heparin is governed by the USP and other national pharmacopeia. Bioanalyses include a routine in vitro coagulation assay and a test for bacterial endotoxins (pyrogens). The heparin assay, when expressed in units/mg or international units/mg, is the amount of heparin that will cause 1 ml of sheep plasma to half-clot when kept for 1 hour at 20°C compared to a USP reference standard (K-4) or the Fifth International Standard for Unfractionated Heparin (WHO-5), respectively. The analysis of LMWHs is governed by the European Pharmacopeia and proposals have also been made to amend the USP monograph to cover LMWHs.[41] Such analyses include in vitro amidolytic assays in a purified system for anti-factor Xa and anti-factor IIa activities, and structural identification by nuclear magnetic resonance and size exclusion chromatography.

Less routine analyses rely on in vitro protein (i.e., AT) binding assays and protein interaction studies, sophisticated ex vivo or in vivo animal assays and finally, human clinical evaluation.[3–7,36,59] Refined chemical, chromatographic, electrophoretic, and spectroscopic assays have been developed that are often used by research laboratories studying heparin. The presence of unsubstituted glucosamine can be measured in heparin and LMWHs by chemical modification with an amine reactive fluorescent reagent.[60] While chemical assays for uronic acid glucosamine have been used in heparin analysis, these have been largely displaced by rapid and more reliable spectroscopic analysis.[61] Chromatographic analysis of heparin and LMWHs primarily involves high-pressure liquid chromatography (HPLC), such as gel-permeation chromatography (GPC) to determine MW and polydispersity.[28,29] This method, once requiring the tedious production of MW standards, can now be performed using a heparinase-prepared LMWH standard through the clever use of dual mass and absorbance detection.[20,62] Ion exchange chromatography, following sample pyrolysis, can be accurately used to determine the level of sulfation or the presence of contaminating metals or anions.[63] Disaccharide compositional analysis (Table 3) and oligosaccharide mapping of heparin and LMWHs have in the past relied on HPLC analysis following the complete or partial heparinase catalysis of nitrous acid depolymerization of heparin or LMWHs.[51,64] Capillary electrophoresis (CE) has gained popularity, making disaccharide analysis and oligosaccharide mapping extremely rapid and sensitive.[65,66] Oligosaccharide mapping by CE can provide data on the content of AT binding sites in different lots of a given LMWH.[67] Polyacrylamide gel electrophoresis has been used to determine the MW and polydispersity of heparin and LMWHs.[68] Spectroscopic analysis, while requiring expensive instrumentation, can facilitate the high resolution analysis of heparin and LMWHs, often providing information that is not available using any other techniques. Proton nuclear magnetic resonance (NMR) spectroscopy has been used to determine the ratio of iduronic to glucuronic acid, the content of an *N*-acetylglucosamine, and position of sulfation.[20,30,59,60] Since this method is quantitative and nondestructive, it can be conveniently used to afford a disaccharide analysis.[30] Proton NMR has also replaced more tedious methods for the determination of contaminating GAGs, such as dermatan sulfate,[35] or the contaminating organic molecules that might be used in the manufacturing of a pharmaceutical grade heparin or LMWH.[69] Finally, proton NMR is extremely valuable in the conformational analysis of heparin.[21,22] Carbon NMR spectroscopy has also been used in heparin and LMWH analysis. While requiring substantially more sample than proton NMR, this method results in higher signal resolution and offers abundant structural information.[69] By measuring the intensity of signals resulting from the reducing end anomeric carbon and all other anomeric carbons, this method offers a sophisticated standard-free determination of the number-averaged MW of heparin[70] and LMWH.[71] While modern

TABLE 3. Disaccharide Composition of LMWHs[51]

	Trisulfated Disaccharides		*Disulfated Disaccharides*		*Monosulfated Disaccharide*
Sample	*2SNS6S*	*NS3S6S*	*NS6S*	*2SNS*	*6S*
Heparin	51.9	1.4	4.1	2.6	1.1
Fractionated LMWH	34.7	1.2	4.0	1.6	0.9
Ardeparin Sodium	86.1	1.7	7.0	6.1	1.3
Dalteparin Sodium	49.3	2.6	3.2	3.1	2.0
Enoxaparin Sodium	71.3	1.9	11.4	2.5	1.4
Nadroparin Calcium	17.2	0.8	1.2	1.8	0.7
Parnaparin Sodium	48.4	0.4	3.6	4.4	0.3
Tinzaparin Sodium	88.9	1.9	7.5	5.4	1.4

soft ionization techniques available in mass spectrometry (MS) offer some intriguing possibilities for heparin and LMWH analysis, the only successful application of MS has been the characterization of heparin-derived oligosaccharides. Fast atom bombardment (FAB),[72] MS/MS-FAB,[73] electrospray ionization (ESI),[74] and matrix-assisted laser desorption ionization (MALDI)[75] have all proved effective in such analyses. CE or HPLC/MS interfaces also offer possibilities for improved, standard-free analysis of heparin disaccharides and oligosaccharides. The primary limitation for the direct MS analysis of heparin or LMWHs remains their high polydispersity and microheterogeneity. Other spectral techniques, such as laser light scattering for determining MW,[76] circular dichroism for determining chiral properties[77] and infrared or Raman spectroscopy[76] for determining functional groups, have been applied to the analysis of heparin.

Special properties unique to a particular LMWH are often analyzed. For example, LMWHs having a UV-active chromophore can be analyzed by spectroscopy.[55,62] Alternatively, the presence of residual reagent (i.e., nitrous acid or nitrite) used in the preparation of a LMWH can be determined chromatographically.[54] Excipients present in a particular formulation, such as bisulfite antioxidant or benzyl alcohol preservative, are generally measured using standard pharmaceutical methods.[78]

STRUCTURAL DIFFERENCES BETWEEN LMWHS

There are eight LMWHs approved for clinical use throughout the world (Table 2). All are prepared through the chemical or enzymatic depolymerization of heparin and have average MW ranges from 3000 to 7000 with polydispersities ranging from 1.1 to 1.5.[7,28] These LMWH drugs also exhibit an enhanced anti-factor Xa to anti-factor IIa activity ratio of greater than 1, ranging from 1.5 to 4.[3–7] Thus, these LMWHs display many similar physical, chemical, and biological properties. A close examination of their structure, however, suggests some significant differences between these products. Polyacrylamide gel electrophoresis, useful for qualitative comparison of mixtures, shows substantial differences between the oligosaccharide and polysaccharide components present in these LMWHs (Figure 3). Disaccharide compositional analysis also shows differences between each product (Table 3), but these differences are no greater than those observed for different preparations of pharmaceutical grade heparins (Table 1).[18,51] Thus, while the LMWHs contain chains of many different structures, their overall disaccharide compositions are fairly similar. It should be noted that LMWH prepared oxidatively using hydrogen peroxide contains a slightly reduced content of unsulfated uronic acids consistent with the proposed mechanism of depolymerization. In addition, the degree of sulfation of this LMWH is also slightly higher than other LMWHs (Table 3).[51]

Detailed analyses of various LMWHs by proton and carbon NMR reveal significant differences in their structures, particularly their end-group residues.[65] LMWHs prepared by deaminative depolymerization contain an anhydromannitol residue at their reducing end (Fig. 2). Indeed, oligosaccharide analysis of a LMWH, prepared using nitrous acid, showed a tetrasaccharide containing this unusual reducing end residue.[51] Heparinase catalyzes β-eliminative cleavage resulting in a 2-*O*-sulfo-unsaturated uronate residue at the nonreducing end, consistent with the known eliminative mechanism of this enzyme.[45,46] Chemical β-elimination is somewhat less specific, yielding both unsulfated and 2-*O*-sulfo unsaturated uronate residues of the nonreducing end (Fig. 2). While there is some speculation regarding

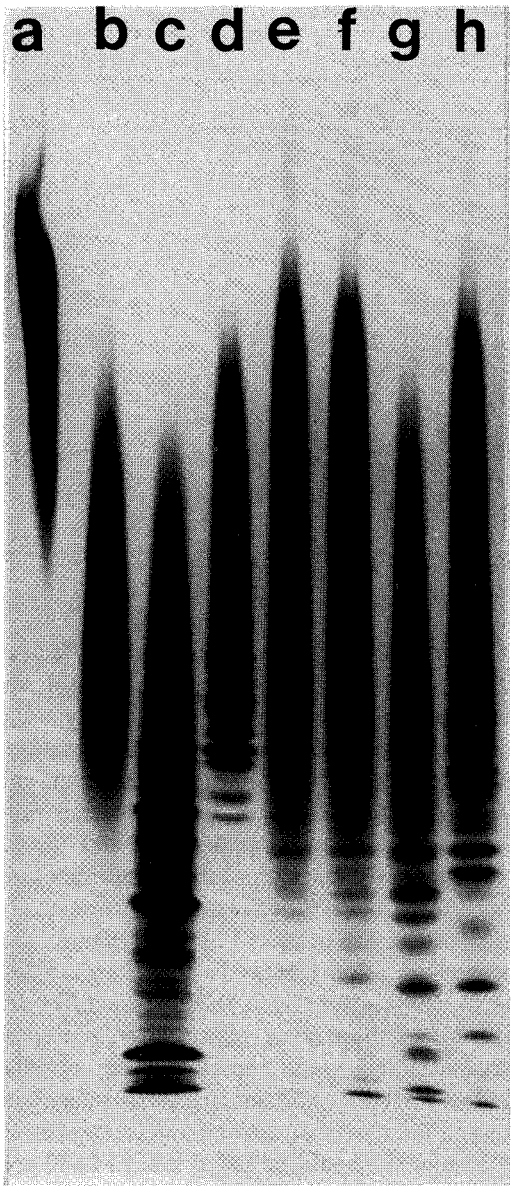

FIG. 3. Polyacrylamide electrophoresis for the analysis of LMWHs. The polyacrylamide gel used to analyze 200 μg samples is a 12 to 22% gradient gel stained with alcian blue. The lanes contain: (a) porcine intestinal heparin; (b) LMW prepared by GPC size fractionation; (c) Nadroparin calcium; (d) Dalteparin sodium; (e) Parnaparin sodium; (f) Ardeparin sodium; (g) Enoxaparin sodium; and (h) Tinzaparin sodium.

the presence of ring-opened, internal residues within product chains in LMWH prepared oxidatively with hydrogen peroxide,[51] it is uncertain whether any unusual saccharide residues are present.

In summary, the type of heparin chosen to be degraded (source and tissue) and the degree of purification of the starting material all contribute to the final characteristics of a LMWH. There are distinct structural differences between LMWHs prepared by deaminative depolymerization, oxidative depolymerization, and β-eliminative depolymerization. These structural differences are associated with the presence or absence of unusual saccharide residues that are artifacts of these depolymerization processes. The structural differences between LMWHs prepared through the same mechanism (i.e., nitrous acid and isoamylnitrate or chemical β-eliminative and enzymatic β-eliminative cleavage) are more subtle. Finally, there are also structural differences between LMWHs prepared through identical methods by two different manufacturers using different processes (i.e., dalteparin vs. nadroparin, both produced by using nitrous acid) (Table 2).

PROCESS VARIABLES IN LMWH PREPARATON-PRODUCT UNIFORMITY

The control of a process for preparing a particular LMWH often represents a challenge (Fig. 4). The most difficult process variable to control is often the quality and identity of the precursor, pharmaceutical grade heparin. This is a particularly complex problem because: (1) heparin is a complex mixture; (2) the USP requirements put few restrictions on the structure of heparin; and (3) steps in the manufacture of heparin often take place outside the pharmaceutical industry, on a farm or in a meat processing facility where there is less regulatory control.

The commercial manufacture of heparin has, in the past, used either bovine lung tissue or porcine intestinal tissue. With the appearance of bovine spongiform encephalopathy ("mad cow disease") and its apparent link to the similar prion-based Creutzfeldt-Jakob disease in humans,[79] the use of bovine tissue products as injectable pharmaceuticals has declined. Bovine lung heparin can be distinguished from porcine intestinal heparin because it contains a structural variant of the AT pentasaccharide binding site (Fig. 1) and other differences in disaccharide composition (Table 1).[19] It is somewhat more difficult to distinguish bovine intestinal heparin or ovine heparin (sheep also carry the scrapie prion). Moreover, blends of pharmaceutical grade heparins prepared from different species might make the content of nonporcine heparin more difficult to assess. Thus, the LMWH manufacturer must first secure a source of pharmaceutical grade heparin of defined tissue and species origin. Once a source (or multiple sources) of pharmaceutical porcine intestinal heparin is established, the process used in its manufacture needs to be controlled. The portion of the tissue that is being used in the process (i.e., hashed pork guts, whole intestine, intestinal mucosa) needs to be established. Other variables, such as the subspecies of pig, the feed given to the animals, the health of the animals, and seasonal variations, may also play a role in the final quality of the pharmaceutical grade heparin being produced. Furthermore, the process for the purification of pharmaceutical grade heparin from tissue must be well controlled.

Even with a well controlled, high quality source of pharmaceutical grade heparin from porcine intestine, heparin still has substantial inherent structural variabil-

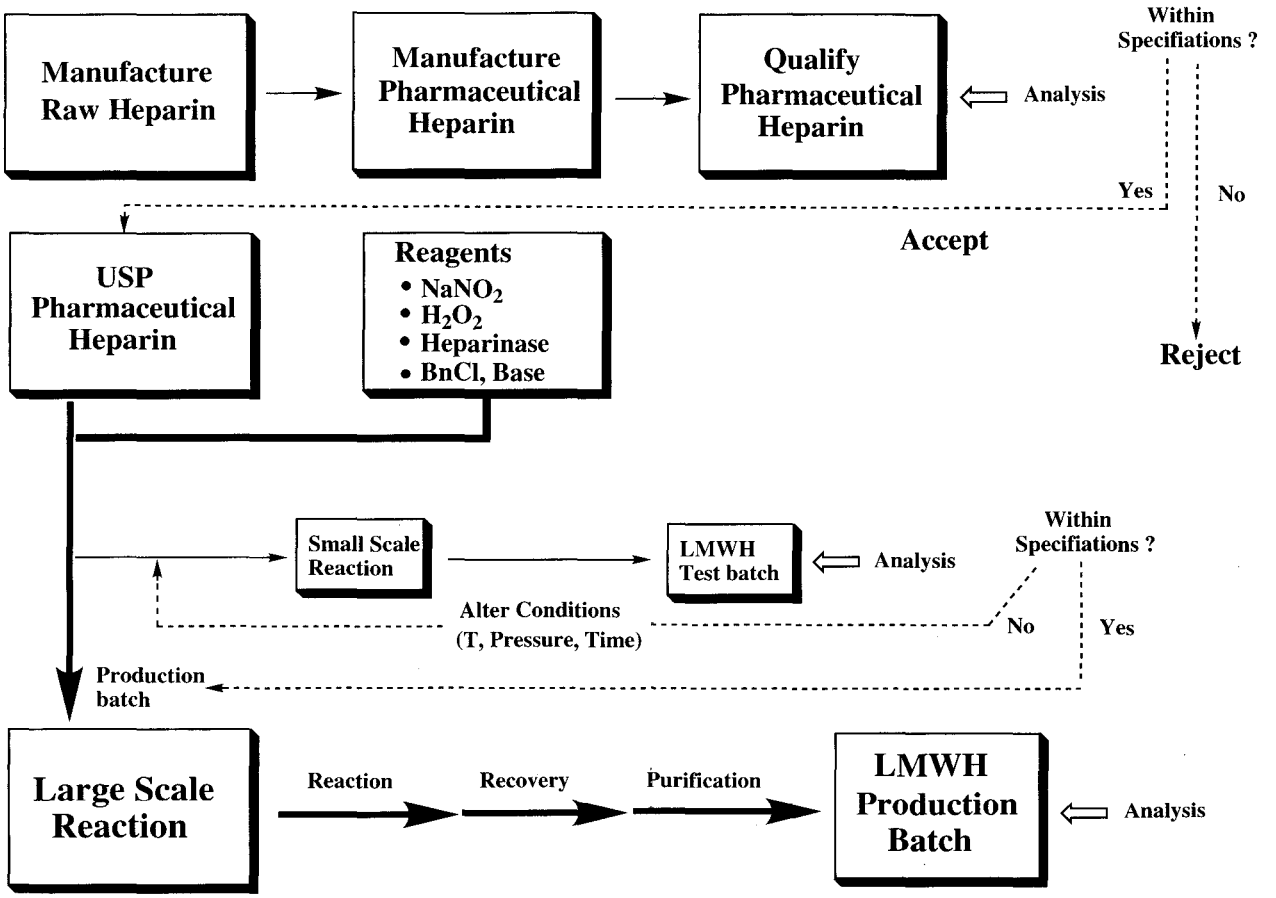

FIG. 4. Process for producing a LMWH. A representative flow chart showing the preparation of a LMWH from raw heparin. A small scale reaction is interposed to test the process and to set the precise conditions to be used in the large scale reaction.

ity (Table 1). This inherent variability of the heparin starting material requires flexibility in the process used to manufacture LMWH. Flexibility in LMWH manufacturing process is handled in several ways. Each LMWH product is defined based on certain specifications set by the manufacturer. These include physical, chemical, and biological properties associated with their drug product. For example, a range of values might be set for average MW (5000 to 6000), average sulfation level (2 to 2.5 sulfate groups/disaccharide) and an anti-factor Xa activity (70 to 80U/mg). Since LMWH is a heterogeneous mixture, these specifications do less to define the final drug product than would a similar set of specifications on a well defined homogeneous pharmaceutical product such as aspirin. To manufacture a LMWH, within product specifications using a fixed process, requires each step to permit some variability in process conditions. For example, in the depolymerization step the permissible temperature might vary by 10°C, the pH by 0.5 units, and the concentrations of reagents used by ±10%. These process variables need to be sufficiently flexible to be used in the manufacturing environment but sufficiently rigid to afford a consistent and uniform LMWH that meets product specifications.

Pharmaceutical companies have been very successful at controlling the manufacturing process for their LMWHs. This is demonstrated by the successful regulatory approval of a number of LMWHs (Table 2). Concerns about the availability of high quality pharmaceutical grade heparin precursor have been addressed by strengthening the relationship between pharmaceutical and meat processing companies. The inherent structural variability in heparin can also be compensated for by performing test runs to calibrate a process within the allowed limits. For example, for a particular pharmaceutical grade heparin that happens to be more sensitive to depolymerization, within a single manufacturing process, it might be possible to reduce the temperature by 1°C (a permissible change within the process parameters) to obtain a LMWH product that falls within specifications.

DIFFERENCES BETWEEN AND WITHIN LMWHS

A LMWH from a particular manufacturer must fall within product specifications to qualify for clinical use. These product specifications are set within a sufficiently

narrow range to ensure a uniform clinical response for a given agent.[3] These specifications, however, are more tolerant than they would be for a homogeneous drug product such as aspirin. Thus, a given LMWH will always have significantly more structural variability, leading to greater biological/clinical variability than exhibited by simpler homogeneous drug products.

Analysis of two LMWHs (Table 3) prepared by similar processes (i.e., dalteparin sodium and nadroparin sodium, both produced using nitrous acid) suggests that they are more similar than two LMWHs produced by different processes but less similar than two batches of a LMWH from a single manufacturer.[3–7] Two LMWHs prepared by chemically related processes (i.e., enoxaparin sodium and tinzaparan sodium, produced by chemical and enzymatic β-elimination, respectively) are structurally related to each other (both contain unsaturated uronate nonreducing ends) but are very different with respect to other physical, chemical, and biological properties.[3–7] Moreover, products prepared by distinctly different processes (i.e., enoxaparin sodium and dalteparin sodium produced by chemical β-eliminative cleavage and by nitrous acid, respectively) are dissimilar in both end-group functionality and physical, chemical, and biological properties.

In summary, there are real and significant structural differences between different LMWHs. The ultimate issue comes down to how structural differences affect the clinical properties of LMWHs. Product uniformity of LMWHs, while not comparable to a structurally defined homogeneous drug substance like aspirin, is certainly sufficient to permit the pharmacologist[80,81] and clinician[3,5] to address the issue of the biological equivalency of these agents.

The authors thank Case VanGorp, Alan Cardin, and Erwin Coyne for their critical reading of this manuscript and helpful suggestions. This work was supported in part by grants GM 38060 and HL 52622 from the National Institutes of Health.

REFERENCES

1. Linhardt RJ, Toida T. Heparin oligosaccharides: New analogues-development and applications. In: Witczak ZJ, Nieforth KA, eds. Carbohydrates in Drug Design. New York: Marcel Dekker; 1997:277–341
2. Linhardt RJ. Heparin: An important drug enters its seventh decade. Chem Indust 1991;2:45–50
3. Green D, Hirsh J, Heit J, et al. Low molecular weight heparin: A critical analysis of clinical trials. Pharmacol Rev 1994;46: 89–110
4. Barrowcliffe TW. Low molecular weight heparin(s). Br J Haematol 1995;90:1–7
5. Donayre CE. Current use of low molecular weight heparins. Semin Vascul Surg 1996;9:362–371
6. Hunt D. Low molecular weight heparins in clinical practice. Southern Medical J 1998;91:2–10
7. Fareed J, Jeske W, Hoppensteadt D, Clarizio R, Walenga JM. Low molecular weight heparins: Pharmacologic profile and product differentiation. Am J Cardiol 1998;82:3L-10L
8. Rosenberg RD. Biochemistry and pharmacology of low molecular weight heparin. Semin Hematol 1997;34:2–8
9. Rosenberg RD, Damus PS. The purification and mechanism of action of human antithrombin-heparin cofactor. J Biol Chem 1973;248:6490–6505
10. Lindahl U, Lidholt K, Spillmann D, Kjellen L. More to heparin than anticoagulation. Thromb Res 1994;75:1–32
11. Fussi F. Process for obtaining low molecular weight heparins endowed with elevated pharmacological properties and products so obtained. US patent #4281108, 1981
12. Lindahl U, Backstrom GE, Thunberg J, et. al. Heparin fragments having selective anticoagulant activity. US patent #4303651, 1981
13. Barnett WE. Anticoagulant substance. US patent #4351938, 1982
14. Loremeau J-C, Choay J, Petitou M. Oligosaccharides having anti-Xa activity and pharmaceutical compositions containing them. US patent #4401662, 1983
15. Langer RS, Linhardt RJ, Cooney CL, Fitzgerald G, Grant A. Heparinase derived anticoagulants. US Patent #4396762, 1983
16. Lindahl U, Backstrom G, Thunberg L, Leder IG. Evidence for a 3-*O*-sulfated D-glucosamine residue in the antithrombin-binding sequence of heparin. Proc Natl Acad Sci (USA) 1980; 77:6551–6555
17. Atha DH, Lormeau J-C, Petitou M, Rosenberg RD, Choay J. Contribution of monosaccharide residues in heparin binding to antithrombin III. Biochemistry 1985;24:6723–6729
18. Linhardt RJ, Rice KG, Kim YS, et al. Mapping and quantification of the major oligosaccharide components of heparin. Biochem J 1988;254:781–787
19. Loganathan D, Wang HM, Mallis LM, Linhardt RJ. Structural variation in the antithrombin III binding site region and its occurrence in heparin from different sources. Biochemistry 1990;29:4362–4368
20. Pervin A, Gallo C, Jandik KA, Han X-J, Linhardt RJ. Preparation and structural characterization of large heparin-derived oligosaccharides. Glycobiology 1995;5:83–95
21. Gatti G, Casu B, Hamer GK, Perlin AS. Studies on the conformation of heparin by 1H and 13CNMR spectroscopy. Macromol 1979;12:1001–1007
22. Mulloy B, Forster MJ, Jones C, Davies DB. NMR and molecular-modelling studies of the solution conformation of heparin. Biochem J 1993;293:849–858
23. Mikhailov D, Linhardt RJ, Mayo KH. NMR solution conformation of heparin-derived hexasaccharide. Biochem J 1997;328:51–61
24. Faham S, Hileman RE, Fromm JR, Linhardt RJ, Rees DC. Heparin structure and interactions with basic fibroblast growth factor. Science 1996;271:1116–1120
25. Hileman RE, Fromm JR, Weiler JM, Linhardt RJ. Glycosaminoglycan-protein interaction: Definition of consensus sites in glycosaminoglycan binding proteins. BioEssays 1998;20:156–167
26. Conrad HE. Heparin-Binding Proteins. San Diego, CA: Academic Press;1998:1–527
27. Jackson RL, Busch SJ, Cardin AD. Glycosaminoglycans: Molecular properties, protein interactions, and role in physiological processes. Pharmacol Rev 1991;71:481–539
28. Ahsan A, Jeske W, Hoppensteadt D, Lormeau JC, Wolf H, Fareed J. Molecular profiling and weight determination of heparins and depolymerized heparins. J Pharm Sci 1995;84:724–727
29. Mulloy B, Gee C, Wheeler SF, et al. Molecular weight measurements of low molecular weight heparins by gel permeation chromatography. Thromb Hemost 1997;77:668–674
30. Griffin CC, Linhardt RJ, VanGorp CL, et al. Isolation and characterization of heparan sulfate from crude porcine intestinal mucosa peptidoglycan heparin. Carbohydr Res 1995;276:183–197
31. Yamada S, Sugahara K. Structure of oligosaccharides isolated from heparan sulfate/heparin and substrate specificities of the degrading enzymes of bacterial origin. Trends Glycosci Glycotechnol 1998;10:95–123

32. Salmivirta M, Lidholt K, Lindahl U. Heparan sulfate: A piece of information. FASEB J 1996;10:1270–1279
33. Gallagher JT, Walker A. Molecular distinctions between heparan sulphate and heparin. Biochem J 1985;230:665–674
34. Marcum JA, Rosenberg RD. Anticoagulantly active heparan sulfate proteoglycan and the vascular endothelium. Semin Thromb Hemost 1987;13:464–474
35. Neville GA, Mori F, Holme KR, Perlin AS. Monitoring the purity of pharmaceutical heparin preparations by high-field 1H-nuclear magnetic resonance spectroscopy. J Pharm Sci 1989;78: 101–104
36. Edens RE, Fromm JR, Fromm S J, Linhardt RJ, Weiler JM. Two-dimensional affinity resolution electrophoresis demonstrates that three distinct heparin populations interact with antithrombin III. Biochemistry 1995;34:2400–2407
37. Coyne E. Heparin - past, present and future. In: Lundblad RL, Brown WV, Mann KG, Roberts HR, eds. Chemistry and Biology of Heparin. Amsterdam, Holland: Elsevier North Holland; 1981:9–17
38. Vidic H-J. Process for the preparation of heparin. US patent #4283530, 1981
39. Okuyama T. Yoshida K, Sakuraik M, et al. Method of separating and recovering mucopolysaccharides from connective tissue of animals. US patent #3862003, 1975
40. Williams RE. Process for the recovery of heparin. US patent #3337409, 1967
41. European Pharmacopeia Commission. Pharmeuropa 1991;3: 161–165
42. Coyne E. From heparin to heparin fractions and derivatives. Semin Thromb Hemost 1985;11:10–12
43. Branelllec JF, Espego J, Picart P. Purified heparin fractions, method for obtaining them and pharmaceutical compositions containing them. US patent #5599801, 1997
44. Jandik K, Kruep D, Cartier M, Linhardt RJ. Accelerated stability studies of heparin. J Pharm Sci 1996;85:45–51
45. Lohse DL, Linhardt RJ. Purification and characterization of heparin lyases from *flavobacterium heparinum*. J Biol Chem 1992;267:24347–24355
46. Linhardt RJ. Analysis of glycosaminoglycans with polysaccharide lyases. In: Varki A, ed. Current Protocols in Molecular Biology, Analysis of Glycoconjugates, Boston, MA: Wiley Interscience; 1994:V 2, 17.13.17–17.13.32
47. Mardiguian JS. Heparin esters and process for their preparation. US patent #4440926, 1984
48. Fussi F. Process for obtaining low molecular weight heparins. European Patent GB2, 002, 406B, 1982
49. De Ambrosi L, Recchia W, Ferrari G. Process for the controlled preparation of low molecular weight glycosaminoglycans. US patent #4987222, 1991
50. Jeske W, Iqbal O, Gonnela S, et. al. Pharmacologic profile of a low molecular weight heparin depolymerized by γ-irradiation. Semin Thromb Hemost 1995;21:201–211
51. Linhardt RJ, Loganathan D, Al-Hakim et al. Oligosaccharide mapping of low molecular weight heparins: Structure and activity differences. J Med Chem 1990;33:1639–1645
52. Fussi F, Fedeli GF. Oligo-heteropolysaccharides a activite semblable a celle de l'heparine, procede pour leur preparation et compositions therapeutiques correspondantes. French patent # FR 78 23499, 1978
53. Lormeau J-C, Petitou M, Choay J. Oligosaccharides having anti-Xa activity and pharmaceutical compositions containing them. US patent #RE 35770, 1998
54. Lormeau J-C, Petitou M, Choay J. Method for obtaining biologically active mucopolysaccharides of high purity by controlled depolymerization of heparin. US patent #5019649, 1991
55. Nielsen JI. Process of using light absorption to control enzymatic depolymerization of heparin to produce low molecular weight heparin. US patent #5106734, 1992
56. Piani S, Tamagnone G, Alpino RR, Milani MR. Heparin derivatives and process for their preparation. US Patent #5010063, 1991
57. Lopez, LL. Depolymerization method of heparin. US patent #4981955, 1991
58. Uzan A. Sulfated polysaccharides obtained from heparin, preparation process, pharmaceutical composition and use thereof. US patent #5849721, 1998
59. Toida T, Hileman RE, Smith AE, Vlahova PI, Linhardt RJ. Enzymatic preparation of heparin oligosaccharides containing antithrombin III binding sites. J Biol Chem 1996;271:32040–32047
60. Toida T, Yoshida H, Toyoda H, et al. Structural differences and the presence of unsubstituted amino groups in heparan sulphates from different tissues and species. Biochem J 1997;322: 499–506
61. Linhardt RJ. Chemical and enzymatic methods for the depolymerization and modification of heparin. In: Ogura H, Hasegawa A, Suami T, eds. Carbohydrates-Synthetic Methods and Applications in Medicinal Chemistry. Tokyo: Kodansha/VCH Publishers; 1992:385–401
62. Van Dedem G, Nielsen JI. Determination of the molecular mass of low molecular mass (LMM) heparin. Pharmeuropa 1991;3: 202–225
63. Rice KG, Kim YS, Grant AC, Merchant ZM, Linhardt RJ. High-performance liquid chromatographic separation of heparin derived oligosaccharides. Anal Biochem 1985;150:325–331
64. Guo Y, Conrad H. The disaccharide composition of heparins and heparan sulfates. Anal Biochem 1989;176:96–104
65. Pervin A, Al-Hakim A, Linhardt RJ. Separation of glycosaminoglycan-derived oligosaccharides by capillary electrophoresis using reverse polarity. Anal Biochem 1994;221:182–188
66. Desai UR, Hoppensteadt D, Fareed J, Linhardt RJ. Measurement of the antithrombin III binding sites in low molecular weight heparins using ^{13}C NMR and capillary electrophoresis. Pharm Sci 1995;1:349–353
67. Desai UR, Wang HM, Ampofo SA, Linhardt RJ. Oligosaccharide composition of heparin and low molecular weight heparins by capillary electrophoresis. Anal Biochem 1993;213: 120–127
68. Edens RE, Al-Hakim A, Weiler JM, Rethwisch DG, Fareed J, Linhardt RJ. Gradient polyacrylamide gel electrophoresis for determination of the molecular weights of heparin preparations and low molecular weight heparin derivatives. J Pharm Sci 1992;81:823–827
69. Casu, B. Structure and biological activity of heparin. Adv Carbohydr Chem Biochem 1985;43:51–134
70. Desai UR, Linhardt RJ. Molecular weight of heparin using ^{13}C nuclear magnetic resonance spectroscopy. J Pharm Sci 1995; 84:212–215
71. Desai UR, Linhardt RJ. Molecular weight of low molecular weight heparins by ^{13}C nuclear magnetic resonance spectroscopy. Carbohydr Res 1994;255:193–212
72. Mallis LM, Wang HM, Loganathan D, Linhardt RJ. Sequence analysis of highly sulfated heparin-derived oligosaccharides using fast atom bombardment mass spectrometry. Anal Chem 1989;61:1453–1458
73. Lamb DJ, Wang HM, Mallis LM, Linhardt RJ. Negative ion fast-atom bombardment tandem mass spectrometry to determine sulfate and linkage position in glycosaminoglycan-derived disaccharides. J Am Soc Mass Spectrom 1992;3:797–803
74. Toida,T, Linhardt,RJ. Structural analysis of heparan sulfate and heparan sulfate oligosaccharides. Trends Glycosci Glycotechnol 1998;10:125–136
75. Rhomberg AJ, Ernst S, Sasisekharan R, Biemann K. Mass spectrometric and capillary elctrophoretic investigation of the enzymatic degradation of heparin-like glycosaminoglycans. Proc Natl Acad Sci (USA) 1998;95:4176–4181

76. Atha DH, Coxon B, Reipa V, Gaigalas AK. Physicochemical characterization of low molecular weight heparin. J Pharm Sci 1995;84:360–364
77. Stone AL. Far-ultraviolet circular dichroism and uronic acid components of anticoagulant deca-, dodeca-, tetradeca-, and octadecasaccharides from heparin fractions. Arch Biochem Biophys 1985;236:342–353
78. Wade A, Weller PJ. eds. Handbook of Pharmaceutical Excipients. Washington, DC: American Pharmaceutical Association 1994; 1–651
79. Schonberger LB. New variant Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. Infect Disease Clinics North Am 1998;12:111–21
80. Fareed J, Jeske W, Hoppensteadt D, Clarizio R, Walenga JM. Are the available low molecular weight heparin preparations the same? Semin Thromb Hemost 1996;22:77–91
81. Brieger D, Dawes J. Production method affects the pharmacokinetic and ex vivo biological properties of low molecular weight heparins. Thromb Haemost 1997;77:317–322