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Review

Preparation and Structure-Activity Differences of Low Molecular Weight Heparins (LMWH)*

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Abstract As a polysaccharide drug, low molecular weight heparins (LMWH) which were introduced as antithrombotic agents about twenty years ago, are now established as surgical thromboprophylaxia drugs and replacing unfractionated heparin (UFH) in the acute treatment of venous thromboemblic disorders. The precise structure of heparin varied with the sources and the preparation methods. The complex structure made the biological activities, for example, antiprotease activity different from each LMWH products, and led to the clinical usage standard different. The production methods, the structure difference and also the antiprotease activity of eight commercial LMWH products were introduced in this paper.

Key words LMWH; production methods; structural difference; antiprotease activity

Heparin[1], a polydispersed and highly sulfated linear glycosaminoglycan (GAG), comprised of alternating 1→4 linked uronic acid and glucosamine residue, had many biological activities. Such as increasing LPL activity[2], it would prevent the proliferation of SMC[3], anti-inflammatory[4], anti-angiogenesis activity[5], and antiviral (HIV-1) activity[6,7] etc. Heparin had been commonly used for prevention and treatment of arterial and venous thrombosis[8]. However, because of complications such as bleeding, thrombocytopenia and osteoporosis, clinicians continued to search for additional anti-thrombotic options. Low molecular weight heparin (LMWH) was developed as a new class of therapeutic agents called antithrombotic

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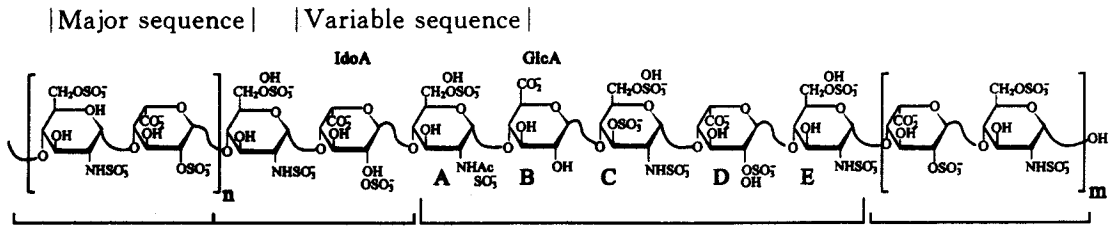
and offered several advantages over the unfractionated heparin (UFH) heparin[9]. LMWH was defined as salts of sulfated GAG having an average molecular weight less than 8000Da. LMWH had a higher anti-factor Xa activity (>70u/mg) and a ratio of anti-factor Xa to anti-factor IIa activity of ≥1.5. Recently, LMWH represented a nearly $2 billion drug class whose application continued to expand globally in various clinical indications. Although heparin had been used clinically as an anticoagulant for the past 65 years, its precise chemical structure remained unknown. In this paper, the chemical structure of heparin, production methods and structural differences of LMWH were introduced.

1 Production and chemical structure of pharmaceutical Heparins

Heparin was synthesized in connective tissue type mast cells as a proteoglycan (PG) molecule and stored in mast cell granules. When mast cells degranulate, heparin was released as GAG heparin. Raw heparin could be separated as peptidoglycan heparin (10%) and GAG heparin (90%). The MW of heparin proteoglycan was approximately 750kD to a million, while the heparin GAG was approximately 5kD to 40kD[10].

Pharmaceutical heparin was prepared from animal tissues that were rich in mast cells such as porcine intestinal mucosal (PIM) and bovine lung (BVL) as a GAG or simply a linear polysaccharide chain without any associated protein. GAG heparin was a heterogeneous polydispersed mixture of sulfated polysaccharide. It was composed of a major trisulfated repeating disaccharide unit, which was α-D-glucosamine-2, 6-disulfate (1→4)-α-L-iduronic acid 2-O-sulfate (75%～90%) and a number of minor disaccharide sequences (10%～25%), which had variable level of O- and N-sulfa groups and glucuronic acid that made heparin structure complex (Figure 1). Larger oligosaccharide sequences corresponded to protein binding sites associated with many biological activities of heparin, for example, heparin contained a specific pentasaccharide sequence that interacted with AT Ⅲ with high affinity, but only about one third of heparin chains contained the AT Ⅲ binding site[11,12].

There were different subspecies of hogs and the mast cell content of intestinal tissue could vary based on the diet and environment in which the animals were raised. These variables potentially contributed to the already complex structure of heparin. Heparin was obtained from different tissues and species and its structure was different. BVL heparin had a higher sulfating level than PIM heparin. The disaccharide composition of PIM heparin also differed substantially from each other. In the past, the commercial manufacture of heparin had used either bovine lung tissue or porcine intestinal tissue, but with the appearance of bovine spongiform encephalopathy (mad cow disease) and its apparent link to the similar prion based disease in human, the use of bovine tissue product as injectable pharmaceutical drug had declined.



Trisulfated disaccharide Disulfated disaccharide Antithrombin Pentasaccharide Binding Site Trisulfated disaccharide

n+m=16 for MW 12,000

Figure 1 The representative chemical structure of pharmaceutical heparin[12]

2 The production methods of LMWH

LWMH were usually acquired from commercial GAG heparin through the controlled, partial chemical or enzymatic depolymerization. The native heparin, which had the MW approximately 15kD, was depolymerized by chemical or enzymatic digestion and then underwent isolation process to get LMWH. Commercial LMWH had different MW that varied from 4kD to 6.5kD, and usually one sixth to one forth of the chains contain AT Ⅲ binding site. There were several methods for preparation of LMWH, that is, oxidative depolymerization with H_2O_2, deaminative cleavage with HNO_2 or isoamyl nitrite, β-eliminative cleavage with benzylation followed by alkaline depolymerization, and cleavage with heparinase[13~15]. Heparin could be oxidative broken down using a variety of oxygen containing reagents like hydrogen peroxide or by ionizing γ-irradiation. Each oxidative method depended on the generation of oxygen radicals to act by oxidizing sensitive saccharide residues with in the heparin polymers. The deanimative method can form an 6-sulfated anhydromannose residue at the reducing terminus, and this residue could be converted to 6-sulfated anhydromanitol (M6S) using a reducing agent such as $NaBH_4$ for stabilization[16]. Twoβ-eliminative methods, one enzymatic and the other chemical, were used in commercially preparing LMWH. In the enzymatic method, heparinase was used to depolymerize heparin. Heparinase usually splited glycoside bonds between N-sulfated glucosamine and iduronic acid 2-sulfate, producing fragments terminus with 4,5-unsaturated iduronic acid 2-sulfate (△UA2S) at the non-reducing end and 2-N-sulfa-3,6-disulfated glucosamine (GlcNp2S3S6S) at the reducing end. Removing or inactivating the enzyme could stop the depolymerization. Chemical β-elimination involved in the direct treatment of heparin with base made LMWH contain an unsaturated uronate residue in the non-reducing end. Cleavage occured specifically at iduronic acid without preference for the presence or absence of a 2-O-sulfa group. Either chemical or enzymatic degradation of heparin could produce specific changes in heparin. The modification of the end groups, internal structures, the change of density and the degree of desulfation during manufacturing could make all the products different each other.

3 Structure difference and antiprotease activity of LMWH

Up to now, eight LMWH approved for clinic had been used throughout the world, and most of them are currently manufactured by chemical depolymerization methods, for example, Ardeparin sodium (USA) was prepared by oxidative depolymerization with hydrogen peroxide, Certoparin sodium (Germany) was prepared by deaminative cleavage with isoamyl nitrite, Nadroparin calcium (France) was prepared by nitrous acid, Enoxaparin sodium (USA) was prepared by β-elimination of benzyl ester of heparin treated by alkaline and so on with the exception of Tinzaparin sodium, which was prepared by using heparinase. These LMWHs displayed many similar physical, chemical, and biological properties. However a close examination of their structure suggested some significant differences, which exhibited in both of their structural and functional properties. From the results of gradient PAGE analysis (Figure 2), all the LMWH products had different MW profile (2000～8000). Beside differences in their MW distribution, the LMWH also exhibited significant variations in their structural characteristics, the content of trisulfated, disulfated, and nonosulfated disaccharide were different (Figure 2 and Table 1)[17~19].

Table 1 Disaccharide composition of LMWH[20]

Sample	2SNS6S/NS3S6S	NS6S/2SNS	6S
UFH	51.9/1.4	4.1/2.6	1.1
A	34.7/1.2	4.0/1.6	0.9
B	88.9/1.8	7.5/5.4	1.4
C	71.3/1.9	11.4/2.5	1.4
D	86.1/1.7	7.0/6.1	1.3
E	48.4/0.4	3.6/4.4	0.3
F	49.3/2.6	3.2/3.1	2.0
G	17.2/0.8	1.2/1.8	0.7

The capital letters of A, B, C, D, E, F, G in column one have the same meaning as in figure 2. 2SNS6S, △UAp2S(1→4)-α-D-GlcNp2S6S; NS3S6S, △UA(1→4)-α-D-GlcNp2S3S6S; NS6S, △UA(1→4)-α-D-GlcNp2S6S; 2SNS, △UAp2S(1→4)-α-D-GlcNp2S; 6S, △UA(1→4)-α-D-GlcNp6S.

From the ^{13}C-NMR spectra (Figure 3) and the disaccharide composition analysis (Table 1), it was known that different method could produce different LMWH, the contents of disaccharide and the negative charge (sulfate groups) were highly different. The HNO_2 and heparinase cleaved the heparin chains only at the glycosidic bonds between the aminosugar and the uronic acid residues and other method, such as H_2O_2 involved in the cleavage between uronic acid and glucosamine residues. The study of these structure activity relationships was not available at this time. It was certainly true that each of the manufacturers used specific patent protected methods and the resulting products exhibited specific characteristics that

distinguished each other, and the structures of LMWH prepared by different depolymerization were distinct.

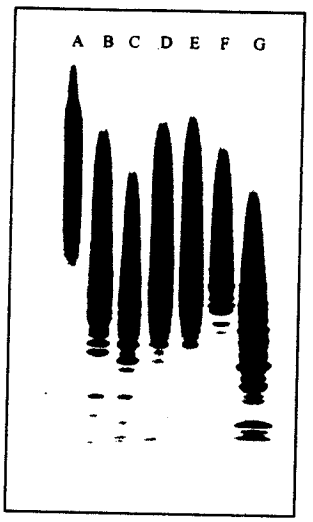


Figure 2 Gradient PAGE analysis of LMWH
(A) Fractioned heparin; (B) Tinzaparin;
(C) Enoxaparin; (D) Ardeparin; (E) Parnaparin
(F) Dalteparin; (G) Nadroparin

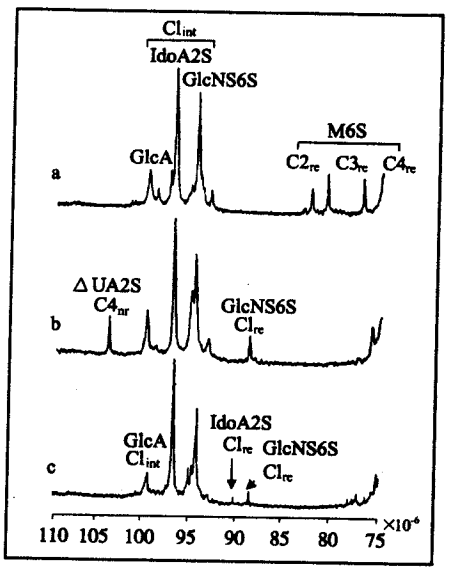


Figure 3 ^{13}C-NMR spectra of LMWH obtained by different methods with (a) HNO_2; (b) Heparinase; and (c) H_2O_2; showing signals associated with typical end-residuals

The antiprotease and anticoagulant activities were very important index of LMWH. The anti-Xa, anti-Ⅱa and their ratio was different between LMWH products (Table2). Compared with UFH, anticoagulant activity of LMWH decreased, but the ratio of anti-Xa / Ⅱa increased. These features made LMWH as a new pharmaceutical drug and was used as an antithrombotic agent in treatment of acute deep vein thrombosis (DVT) and other diseases.

Table 2 Anticoagulant and antiprotease activity between LMWH products[21]

Samples	Dose	Antiprotease Activity (IU/mg)			Mr	Anticoagulant Activity
	(IU)	Anti-Xa	Anti-Ⅱa	Anti-Xa/Ⅱa	(kD)	(IU/mg)
UHF	5000	193	193	1	15	166
Tinzaparin	3000	79	53	1.5	5.8	-
Certoparin	4000	105	25	4.2	5.1	-
Enoxaparin	4000	98	25	3.9	4.4	66
Ardeparin	5000	60	24	2.5	5.9	-
Parnaparin	3075	82	20	4.1	6.5	-
Dalteparin	5000	170	-	2.2	6.0	51
Nadroparin	3075	95	27	3.5	4.8	46
Reviparin	3500	120	-	-	3.9	-

Note: -Not detected

It was known that LMWH had a much lower affinity for plasma matrix proteins, en-

dothelial cells, macrophages and platelet 4 than unfractionated heparin did. The absence of protein binding of the LMWH contributed to a number of potential advantages over heparin. Firstly, LMWH were more stable in dose response and did not require lab monitoring. Secondly, LMWH had better bioavailability when injected subcutaneous in low dose. Thirdly, LMWH had longer plasma half-life and finally, LMWH were associated with a lower incidence of heparin-induced thrombocytopenia and produced less bleeding for equivalent antithrombotic effect etc.

4 Conclusion

As discussed above, LMWH showed remarkable variations in MW, structure and a number of other characteristics. Although these differences may not become apparent at the low doses used for prophylactic indications, but at a higher doses used for therapeutic indications may become much more important. Owing to these differences, LMWH can't be interchanged in clinical usage. The United State FDA and the WHO had suggested that LMWH were not interchangeable drugs. Different products had different physical and chemical compositions, translating into the differences in biological actions, and clinical trials for specific indications on each product were carried out at optimized dosages for each product. Thus, the specific dosages for individual product must be used, and each drug was classified by the FDA as a distinct drug and can't be interchanged. Therefore preclinical trials were necessary to access suitable dose regimens for any given agent in any given indication.

From the economic considerations, LMWH was associated with similar complications as heparin, but the complications occured less frequently. Currently, the main limitation in using LMWH in place of heparin was its cost. The cost for LMWH is 10 to 20 times that of heparin. However, taking into account the cost incurred by hospitalization and long term monitoring of anticoagulation in patients treated with heparin, certain trials had proved the cost of LMWH to be the same or less than the cost of heparin overall.

LMWH was manufactured using a verity of chemical and enzymatic depolymerization procedures that result in products with distinct characteristics and exhibit both structural and functional heterogeneity and each of these agents represents a distinct drug entity, and will have its own safety/efficiency profile, and no standardize potency assignments or bioequivalence studies can be used to interchange agents for a given clinical indication. As a polysaccharide drug, LMWH had already been used in our country to prevent postoperative venous thrombosis in high-risk orthopedic surgery patients[22], acute stroke, ischemic stroke[23], percutaneous transluminal coronary angioplasty (PTCA)[24], myocardial infraction and unstable angina[25] etc. Investigations into the efficacy of LMWH in new indications were ongoing.

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低分子量肝素的制备及其结构与活性差异*

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摘 要 低分子肝素作为一种抗血栓的多糖药物在临床中已应用了二十多年,目前已作为外科预防血栓形成药物,并在治疗急性静脉栓塞紊乱方面取代了未分级肝素。因肝素的来源和制备的方法不同使低分子肝素的精细结构不同,低分子肝素结构的复杂性,使得各产品的生物活性,例如抗蛋白酶活性不同,从而导致其临床使用的标准不同。该文将对低分子肝素的制备方法及其结构和抗蛋白酶活性的差异进行报导。

关键词 低分子量肝素;制备方法;结构差异;抗蛋白酶活性

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