Structure and Activity of a New Low-Molecular-Weight Heparin Produced by Enzymatic Ultrafiltration

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ABSTRACT: The standard process for preparing the low-molecular-weight heparin (LMWH) tinzaparin, through the partial enzymatic depolymerization of heparin, results in a reduced yield because of the formation of a high content of undesired disaccharides and tetrasaccharides. An enzymatic ultrafiltration reactor for LMWH preparation was developed to overcome this problem. The behavior, of the heparin oligosaccharides and polysaccharides using various membranes and conditions, was investigated to optimize this reactor. A novel product, LMWH-II, was produced from the controlled depolymerization of heparin using heparin lyase II in this optimized ultrafiltration reactor. Enzymatic ultrafiltration provides easy control and high yields (>80%) of LMWH-II. The molecular weight properties of LMWH-II were similar to other commercial LMWHs. The structure of LMWH-II closely matched heparin's core structural features. Most of the common process artifacts, present in many commercial LWMHs, were eliminated as demonstrated by 1D and 2D nuclear magnetic resonance spectroscopy. The antithrombin III and platelet factor-4 binding affinity of LMWH-II were comparable to commercial LMWHs, as was its *in vitro* anticoagulant activity. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:1375–1383, 2014

Keywords: low molecular weight heparin; heparin lyase; ultrafiltration; anticoagulant; NMR; mass spectrometry; surface plasmon resonance; polymeric drugs; structure-activity relationship; enzymes

INTRODUCTION

Heparin [also known as unfractionated heparin (UFH)] is a polysaccharide-based anticoagulant drug that was first introduced into clinical practice nearly 100 years ago.[1] Low-molecular-weight heparins (LMWHs), derived from UFH, were more recently introduced, nearly 35 years ago.[1,2] The need for heparin and LMWHs continues to increase as modern medical treatments and procedures, such as the treatment of deep vein thrombosis, postsurgical control of clots,[3,4] extracorporeal therapy (i.e., kidney dialysis and heart–lung oxygenators), and the use of indwelling heparinized catheters and shunts, expand in first-world countries and are introduced in third-world countries.

The major repeating disaccharide unit of heparin is α-L-IdoA2S(1→4)-α-D-GlcNS6S (where IdoA is idopyranosyluronic acid, S is sulfo, and GlcN is 2-deoxy, 2-amino glucopyranose), but the minor disaccharide units are also present with different structural and sulfation patterns that are integral to heparin's major therapeutic activity, the inhibition of coagulation cascade proteases, thrombin (factor IIa) and factor Xa. A well-studied pentasaccharide sequence in heparin having the structure, →4)-α-D-GlcNAc6S(1→4)-β-D-GlcA(1→4)-α-D-GlcNS3S6S(1→4)-α-L-Ido2S(1→4)-α-D-GlcNS6S(1→ (where GlcA is glucopyranosyluronic acid and Ac is acetyl) is critical to heparin's specific activation of the serine protease inhibitor antithrombin III (ATIII). Heparin is able to bind both ATIII and thrombin to afford a ternary complex, inactivating thrombin and thus preventing fibrin clot formation. FXa does not interact directly with heparin but is instead inhibited by heparin–ATIII binary complex. The inactivation of thrombin by ATIII requires the longer heparin chains (>15 saccharide units) common to UFH, whereas small heparin chains (from 5 to 15 saccharide units) common to LMWH are capable of binding only ATIII, inactivating factor Xa. Thus, LMWHs are considered factor Xa-selective anticoagulant/antithrombotic drugs.[1,5]

The major physiologic role of platelet factor-4 (PF4), which is released from the alpha-granules of activated platelets, is to bind and neutralize heparin and heparan sulfate on the endothelial surface of blood vessels, thereby inhibiting local ATIII activation and promoting coagulation. The heparin–PF4 complex is the antigen in heparin-induced thrombocytopenia (HIT), an idiosyncratic autoimmune reaction to the administration of the anticoagulant heparin. LMWHs have a lower binding affinity for PF4 than does heparin, which both improve their anticoagulant activity and reduce their incidence of HIT.[6,7] Finally,

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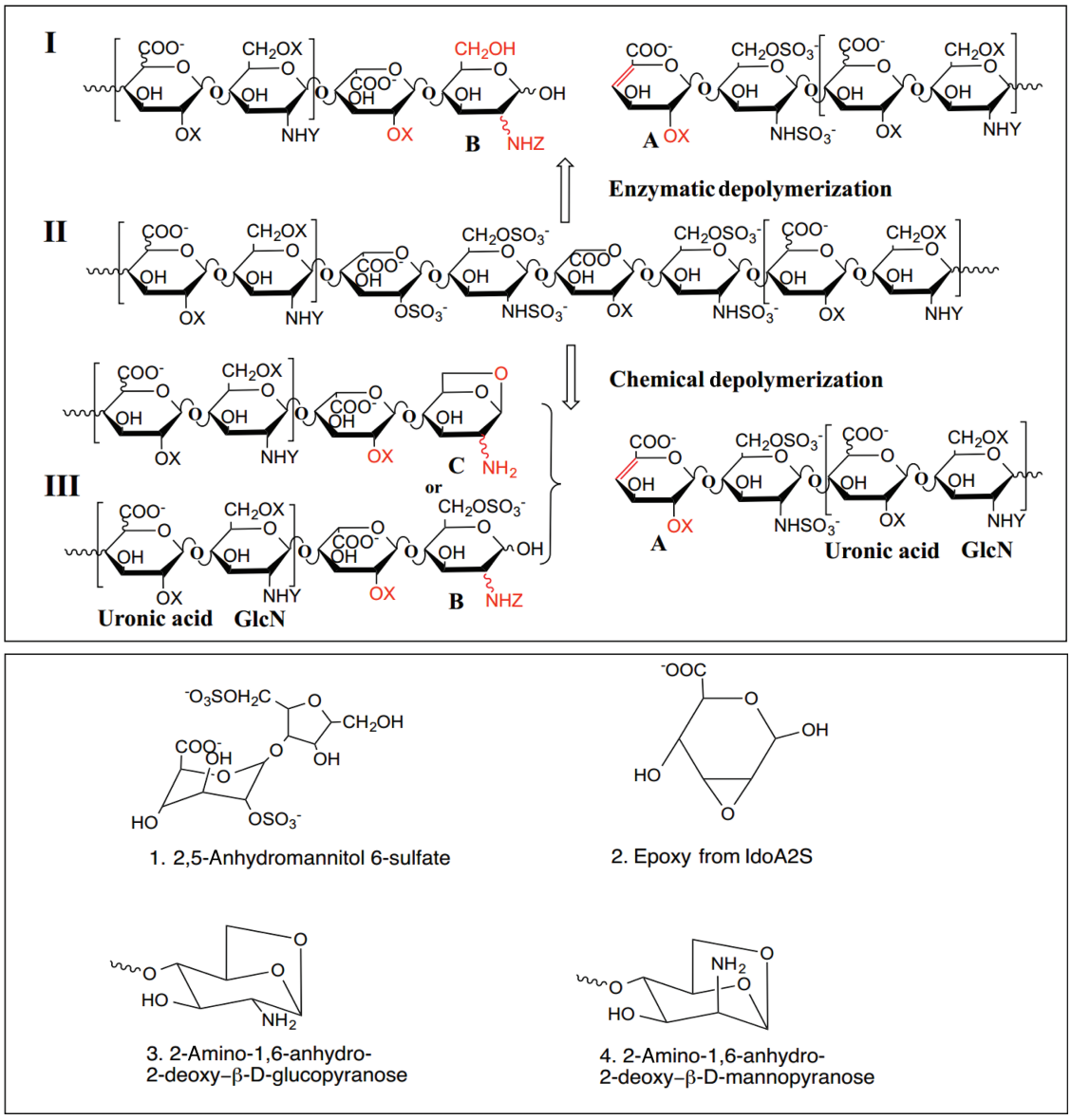

Figure 1. Structural features and process artifacts in LMWHs. Upper panel: Major structures of a LMWH prepared using heparin lyase I, Tinzaparin (I), UFH from porcine intestine (II), and commercial LMWH, Enoxaparin (III). (X = SO_3^- or H; Y = SO_3^- or Ac^-; Z = SO_3^-, Ac^- or H). Lower panel: Process artifacts present in LMWHs prepared using chemical processes.

the enhanced subcutaneous bioavailability and improved pharmacodynamics of LMWHs have increased the clinical use of these anticoagulants in recent years.[1,8]

Currently, the commercial preparation of LMWHs from UFH includes the controlled chemical depolymerization of heparin by peroxidative cleavage, nitrous acid cleavage, and chemical β-elimination (Fig. 1, upper panel, II–III). These chemical methods result in process artifacts including 2,6-anhydromannitol (Fig. 1, lower panel, structure 1), epoxide (Fig. 1, lower panel, structure 2), 1,6-anhydroglucopyranose (Fig. 1, lower panel, structure 3), and 1,6-anhydromannopyranose (Fig. 1, lower panel, structure 4), as a result of harsh reaction conditions that are used in their preparation.[9,10] In contrast, enzymatic depolymerization, a much milder approach, has also been used to make LMWH. Heparin lyase I, isolated from *Flavobacterium heparinum*, is most commonly used to enzymatically depolymerize heparin (Fig. 1, upper panel, II to I).[8,11–13] Previous studies, however, demonstrate that although both heparin lyase I and II can cleave →4)-α-D-GlcNS6S(1→4)-α-L-IdoA2S(1→ linkage, heparin lyase I is highly selective for →4)-α-D-GlcNS3S6S(1→4)-α-L-IdoA2S(1→ and heparin lyase II has selectivity for →4)-α-D-GlcNS6S(1→4)-α-L-IdoA(1→.[14,15]

Heparin lyases can be used under mild conditions (room temperature at physiologic pH) to afford LMWHs (Fig. 1, panel I) with fewer process artifacts generated through side reactions. However, enzymatic depolymerization without chain length control can easily result in the overdigestion of heparin, converting an active LMWH into smaller chains without bioactivity, such as disaccharides and tetrasaccharides. Moreover, the heparin lyases, particularly heparin lyase I, are known to selectivity act at linkages present within the ATIII–pentasaccharide binding site[15] making the loss of anticoagulant activity

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