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REINVESTIGATION OF SYNTHESIS OF ENOXAPARIN UNDER PTC CONDITIONS

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ABSTRACT: Heparin is a mixture of glycosaminoglycan (GAG) chains originating from the porcine intestinal mucosa. It is used therapeutically as an anticoagulant for the treatment and prevention of thrombosis. Glycosaminoglycans such as heparin (H) and heparan sulfate (HS) are considered attractive therapeutic agents because they modulate many biological processes and have been implicated in numerous pathologies, including cardiovascular, cancer, inflammation, metabolic, and neurodegenerative diseases and viral infections. These biological functions are believed to be dependent on the interaction of these linear polysaccharides with key proteins such as growth factors, cytokines, proteases, adhesion proteins, lipid binding proteins, etc., which have a heparin-binding domain in common and are termed heparin binding proteins (HBPs). The variability in unfractionated heparin's pharmacokinetic properties and pharmacological effects led to the development of low MW heparin (LMWH), which is a degraded product of heparin using chemical or enzymatic cleavage techniques. The most common form of LMWH in the U.S. is enoxaparin, which is produced by β-eliminative cleavage of the benzyl esters of porcine mucosal heparin under alkaline conditions. This cleavage process leads to the generation of unnatural structures in enoxaparin. We present herein the purification strategies used to generate hexasaccharide that was further evaluated *in vitro* for their affinity for these protein targets, as well as heparanase inhibition. The hexasaccharide contains the same (L-Iduronic acid, D-Glucosamine) carbohydrate backbone but varying substitution patterns. We present here a new purification process of Enoxaparin with good yield.

INTRODUCTION: The anticoagulant drug Heparin has been used to treat thrombosis for a recommend 80 years [1]. The drug was originally isolated from dog liver and demonstrated to possess anticoagulant activity in 1916 [2].

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During the 1930s, heparin was successfully prepared from bovine lung, and this drug source was later developed as a pharmaceutical product in the United States [3, 4].

Heparin is a linear polysaccharide composed of a repeating disaccharide building block of alternating β-1,4-linked hexuronic acid (Hex-A) and glucosamine residue (Glc-N). The Hex-A can be either β-D-Glucuronic acid (Glc-A) or α-L-Iduronic acid (Ido-A) at which the C-2 position can be substituted by an O-sulfo group.

The Glc-N may be modified by an N-acetyl group (Glc-N-Ac), an N-sulfo group (Glc-N-S), or can be un-substituted, whereas, O-sulfo group substitution can occur at its C-3 and/or C-6 positions [5, 6]. A pentasaccharide sequence of Glc-N-Ac/NS(6S)-Glc-A-Glc-NS(3S,6S)-Ido-A(2S)-Glc-NS(6S) is the structural motif for heparin that specifically binds to antithrombin III (ATIII) and inactivates the blood clotting process [7].

Heparin molecule comes under the family of Glycosaminoglycans (GAGs) and consists of the heterogeneous mixture of the polymer; due to this, the molecule has been referred to as "Unfractionated Heparin (UFH) amongst clinicians and researchers [8]. Heparinpossesses biological functionality towards angiogenesis and host-pathogen interactions [9-11]. Furthermore, Heparin is popular in the pharmaceutical industry for its anticoagulant properties, and its de-polymerized version, termed Low Molecular Weight Heparins (LMWH), has gained much attention in the recent past. Different types of LMWH are derived based on differing de-polymerization methods. These molecules are similar to UFH in monosaccharide composition and oligosaccharide sequence. LMWH possess several advantages over UFH due to their lower molecular mass, including prolonged antithrombotic effect and better bioavailability. Given that LMWH do not bind to plasma proteins and endothelial cells, they have a longer half-life in circulation [12]. Due to the often-reported side effects causing Heparin Induced Thrombocytopenia (HIT), LMWHs have been explored as anticoagulants [13]. These molecules are considered more potent compared to unfractionated heparin (UFH) [14]. Some of authors reported that fondaparinux and danaparoid in patients with suspected HIT [15], anticoagulant treatment, abnormal coagulation parameters and systemic anticoagulation associated with decreased venous thromboembolism in critically ill influenza A H1N1-acute respiratory distress syndrome patient [16-18].

In this paper, we describe the significant procedure for the synthesis of the Enoxaparin, leading to a simplification and shortening of the process. The synthesis steps have been shown in **Scheme 1**. We have used a method for the de-polymerization of Heparin sodium to get Enoxaparin, and have characterized the synthetic intermediates in their production. It is our hope that the data presented here and the convenience of our method will facilitate further investigation of this important class of compounds. The spectral and analytical data strongly supported the structure of heparin.

Experimental Section: Melting points were uncorrected. Infrared spectra were obtained by using a BrukerWM-4(X) spectrometer 577 model. ^{1}H NMR (400MHz) and ^{13}C NMR (100MHz) spectra were recorded on a Bruker WM-400 spectrophotometer in $CDCl_3$ with tetramethylsilane as reference. Mass spectra (ESI) were carried out on a JEOL SX-102 spectrophotometer. Elemental analysis was done on a Carlo Erba EA 1108 automatic elemental analyzer. The chemicals and solvents were commercial grade, used without further purification. Purification of the synthesized compounds by column chromatography and thin-layer chromatography (TLC) was carried out by using alumina sheets purchased from Merck with ethanol as the moving phase.

General Procedure for the Synthesis of Enoxaparin Stages:

Stage 1: Taken heparin sodium (1.0 mol) was completely dissolved in purified water (100 mL)at 30-35 ºC. Check the P^H of reaction mass, should be 6.8 to 7.2 ranges. If the pH range is increases adjusted by using 5N NaOH solution and Sodium sulphite was charged to heparin sodium solution. The reaction mass stirred for 10 minutes at 30-35 ºC. Took another round-bottomed flask catalyst Cetrimide (PTC) was completely dissolved in purified water at 30-35 ºC. The prepared heparin solution was added to cetrimide solution and followed by stirring at 30-35 ºC for 0.5 h, after maintained the reaction mass settled for 3.0 h solid separated was filtered washed with purified water and MDC for two times to get the corresponding compound.

Stage 2: To the round-bottomed vessel added Stage-1 (0.1 mmol) material dissolved in MDC (100 mL) at 35-40 ºC in 1.5h, cool to reaction mass than a solution of benzyl chloride (0.12 mmol) was added. The reaction mass stirred for 18.0 to 40.0 h at 35 ºC. The reaction mass was cooled to room temperature than added MDC and Methanol was added. Sodium acetate was added to reaction mass, followed by water maintained for 0.5 h. Gummy

material was formed decant the organic layer washed with methanol (5 × 20 mL). The solid material dried under vacuum dried at 50-55 ºC.

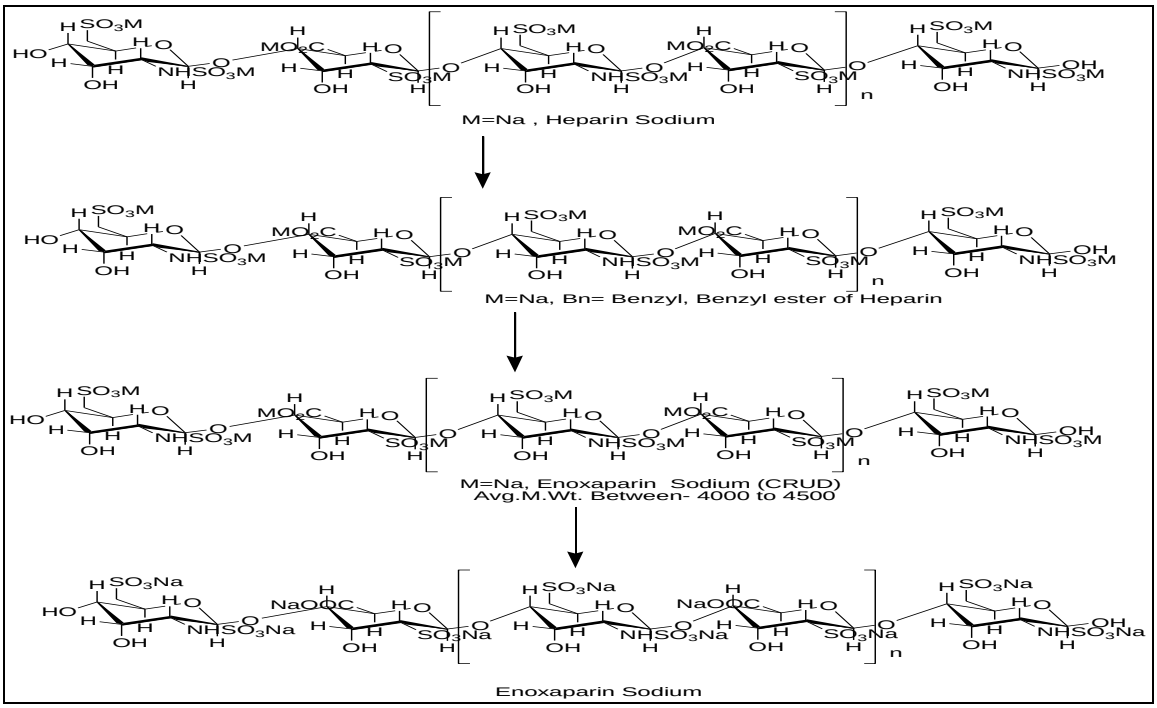
Stage 3: To a stirred solution of Stage-2 (0.1 mmol) compound in purified water added anhydrous Sodium sulphite and EDTA salt was charged. The reaction mass was stirred for 15 min, heated to 60-65 ºC. Added 5N sodium hydroxide solution maintained for 0.5h. The reaction mixture was cooled to 30-40 ºC than adjusted pH neutral by 6N aq. HCl. Ethanol was added to reaction mass and stirrer for 15 minutes settled the reaction mass for 1h, gummy material was formed. The solvent layer was separated and dehydrates with methanol the solid material was filtered and dried under vacuum at 60 ºC.

Stage 4: Stage-3 (0.1 mmol) compound dissolves in purified water (100 mL) adjusted pH by using 5N NaOH solution to 8.0-9.0 range. Heat the reaction mass up to 50 ºC added hydrogen peroxide stirred for 1h. The reaction mass was cooled to 30-35 ºC, check the pH of reaction mass and adjusted by using 5N NaOH solution (pH 6.0-6.5).

Stirred for 10 min and filtered through hyflo bed under Nitrogen gas EtOH was added to the reaction mass and stirred for 15 min. Separate the solvent layer from reaction mass and added MeOH to gummy material to get the purified compound.

Characterization of Enoxaparin: Off white crystalline powder, U.V. 232 nm; ^{1}H NMR (600 MHz): 0.10-2.00; 2.10-3.20; 5.70-8.00; 3.35-4.55; ^{13}C NMR (300 MHz): 176.47, 176.09; 175.66; 170.42, 145.97, 107.099, 103.01, 100.27, 98.57, 98.03, 94.05, 92.36, 79.39, 78.45, 77.01, 76.49, 75.81, 75.44, 74.71, 74.05, 72.24, 71.88, 70.80, 70.35, 69.99, 68.49, 67.59, 64.20, 63.91, 61.05, 59.17, 58.83, 50.31, 49.78, 49.49, 49.20, 48.92, 48.63, 23.29, 18.18. Average molecular weight: 4345 (Range: 3800-5000Da) < 2000Da: 14.5% (Range: 2.0% - 20.0%), 2000 -8000Da: 72.0% (Range:-68 .0% - 82.0%) > 8000 Da: 8.61% (Range:- NMT 18.0%). Anti-factor Xa (on dry basis): 108 IU/mg (Range: 90-125 IU/mg). Anti-factor IIa (on dry basis): 28 IU/mg (Range: 20 -35 IU/mg), The ratio of anti-factor Xa / IIa: 3.857 (Range: 3.3 -5.3) Molar ratio of sulphate ions to carboxylate ions: 2.2 (less than 1.8), pH -10% solution in water: 6.6 (Range: 6.2-7.7). Specific absorbance at 231nm: 15.8% (Range: 14.0-20.0). Loss on drying: 2.48% (not more than 10.0%). Heavy metals (not more than 30 ppm): Compiles.

RESULTS AND DISCUSSION: Routes used to de-polymerization of the compound described in this work are depicted in **Scheme 1.** Key intermediates 2, 3 were prepared from corresponding Heparin sodium 1 by the complexation, separation, reduction.



SCHEME-1

CONCLUSION: We report in this paper the purification strategies used to generate hexa-saccharides that were further evaluated *in-vitro* for their affinity for these protein targets, as well as heparanase inhibition. The hexasaccharides contain the L-Iduronic acid-D-Glucosamine carbohydrate backbone but varying substitution patterns. We present here a new purification process of Enoxaparin with good yield.

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CONFLICTS OF INTEREST: No conflict of interest.

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