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Parent heparin and daughter LMW heparin correlation analysis using LC-MS and NMR

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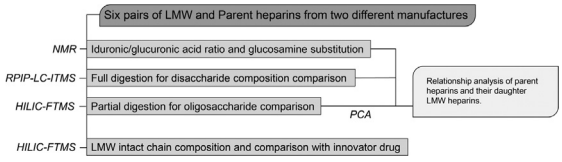
HIGHLIGHTS

- Low molecular weight heparins prepared from different heparin parents were analyzed.
- An integrated analytical approach relied on LC-MS and NMR analysis.
- Monosaccharide compositional analysis relied on top-down NMR analysis.
- Intact chain, oligosaccharide, and disaccharide analyses relied on LC-MS.
- Differences due to parent heparin were observed using principal component analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Heparin is a structurally complex, polysaccharide anticoagulant derived from livestock, primarily porcine intestinal tissues. Low molecular weight (LMW) heparins are derived through the controlled partial depolymerization of heparin. Increased manufacturing and regulatory concerns have provided the motivation for the development of more sophisticated analytical methods for determining both their structure and pedigree. A strategy, for the comprehensive comparison of parent heparins and their LMW heparin daughters, is described that relies on the analysis of monosaccharide composition, disaccharide composition, and oligosaccharide composition. Liquid chromatography-mass spectrometry is rapid, robust, and amenable to automated processing and interpretation of both top-down and bottom-up analyses. Nuclear magnetic resonance spectroscopy provides complementary top-down information on the chirality of the uronic acid residues and glucosamine substitution. Principal component analysis

Abbreviations: LMWH, Low Molecular Weight Heparin; OSCS, Over-sulfated chondroitin sulfate; USP, United States Pharmacopeia; PNNL, Pacific Northwest National Laboratory; API, Active pharmaceutical ingredient; 1D-H^1, One-dimensional Proton; 2D-HSQC, Two-dimensional Heteronuclear Single Quantum Coherence Spectroscopy; NMR, Nuclear magnetic resonance; LC-MS, Liquid chromatography-mass spectrometry; ESI–ITMS, Electrospray ion-trap mass spectrometry; RPIP–LC, Reverse-phase ion-pairing liquid chromatography; HILIC, Hydrophilic interaction chromatography; FTMS, Fourier transform mass spectrometry; dp, Degree of Polymerization; PCA, Principal Component Analysis; PC, Principal Component; NRE, Non-reducing End; RE, Reducing End; TrBA, Tributylamine; 0S, ΔUA (1 → 4) GlcNAc; NS, ΔUA (1 → 4) GlcNS; 6S, ΔUA (1 → 4) GlcNAc6S; 2S, ΔUA2S (1 → 4) GlcNAc; NS2S, ΔUA2S (1 → 4) GlcNS; NS6S, ΔNS2SUA (1 → 4) GlcNS6S; 2S6S, ΔUA2S (1 → 4) GlcNAc6S; TriS, ΔUA2S (1 → 4) GlcNS6S; ΔUA, 4-deoxy-β-L-*threo*-hex-4- enopyranosiduronic acid; GlcN, Glucosamine; IdoA, Iduronic acid; GlcA, Glucuronic acid; Ac, Acetyl; S, Sulfate.

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Nuclear magnetic resonance
Correlation

(PCA) was applied to the normalized abundance of oligosaccharides, calculated in the bottom-up analysis, to show parent and daughter correlation in oligosaccharide composition. Using these approaches, six pairs of parent heparins and their daughter generic enoxaparins from two different manufacturers were comprehensively analyzed. Enoxaparin is the most widely used LMW heparin and is prepared through controlled chemical β-eliminative cleavage of porcine intestinal heparin. Lovenox®, the innovator version of enoxaparin marketed in the US, was analyzed as a reference for the daughter LMW heparins. The results, show similarities between LMW heparins from two different manufacturers with Lovenox®, excellent lot-to-lot consistency of products from each manufacturer, and detects a correlation between each parent heparin and daughter LMW heparin.

1. Introduction

Heparin is a structurally complex mixture of linear anionic polysaccharides with an average molecular weight of 16 kDa and is widely used as a clinical anticoagulant [1]. Low molecular weight (LMW) heparins (average molecular weight 4 kDa to 6 kDa) are prepared through the controlled chemical or enzymatic depolymerization of heparin [2,3]. Due to their improved bioavailability and pharmacodynamics LMW heparins have replaced heparin for many therapeutic applications [4].

Heparin is a natural product, extracted from food animal tissues rich in mast cells, such as porcine intestine [1]. The complex supply chain for the manufacture of heparin and LMW heparins, involving both the food industry and the pharmaceutical industry, has made the sourcing of safe product quite complex [3,5]. Impurities, such as viruses and prions of this food animal product, as well as shortages of animal sourced materials are of growing concern [6,7]. Moreover, it is difficult to distinguish heparin prepared from approved food animal sources, such as porcine intestine, from heparins prepared from non-approved tissues, such as bovine lung or bovine intestine [7,8]. In 2007-8, there was a heparin crisis that resulted in a number of US patient deaths. Heparin, prepared in China, was adulterated with an inexpensive semi-synthetic polysaccharide, over-sulfated chondroitin sulfate (OSCS) [5,9].

As a result of manufacturing regulatory concerns, there have been intensified efforts to develop improved methods for the analysis of both heparin and LMW heparins [10–16]. Our laboratory has contributed methods for disaccharide analysis [17–20], bottom-up analysis [21,22], top-down analysis [23,24] and integrated approaches for heparin analysis [25,26]. One aspect of heparin analysis that has remained relatively unexamined is the relationship between parent heparin and daughter LMW heparin prepared from different manufacturers using variants of a specific manufacturing process.

In the current study, we examine six parent heparins coming from two different manufacturers, one located in the U.S. and one located in Europe. These heparins were each converted to LMW heparin products, generic versions of the innovator drug, Lovenox®, through controlled chemical β-eliminative depolymerization [2,3]. While the exact processes, used in converting a heparin to an enoxaparin, are trade secrets, they generally involve the steps shown in Fig. 1. Slight process differences or differences in heparin starting material might result in structural differences in the resulting LMW heparin product. Generic LMW heparins, presumed to be structurally identical, were compared with the innovator drug and one another and with their heparin parents using an integrated analytical approach. The goal of this forensic study is to understand whether a daughter LMW heparin could be traced to its parent heparin and whether different variations of a manufacturing processes result in structural signatures in LMW heparin products.

2. Experimental section

2.1. Materials

Lovenox®, drug product from Sanofi-Aventis (Bridgewater, NJ) was obtained from commercial suppliers. Six pairs of parent heparin and generic enoxaparin were provided as their active pharmaceutical ingredients (APIs), three pairs form a U.S. based and three from a European based manufacturer. All heparin and LMW heparins were analyzed at the same time prior to their expiration dates. Unsaturated heparin disaccharide (dp2) standards (0S: ΔUA (1 → 4) GlcNAc; NS: ΔUA (1 → 4) GlcNS; 6S: ΔUA (1 → 4) GlcNAc6S; 2S: ΔUA2S (1 → 4) GlcNAc; NS2S: ΔUA2S (1 → 4) GlcNS; NS6S: ΔUA (1 → 4) GlcNS6S; 2S6S: ΔUA2S (1 → 4) GlcNAc6S; TriS: ΔUA2S (1 → 4) GlcNS6S, where ΔUA is 4-deoxy-β-L-*threo*-hex-4-enopyranosiduronic acid, GlcN is glucosamine, Ac is acetyl, and S is sulfate) (Fig. S1) were purchased from Iduron (Manchester, UK). Tributylamine (TrBA) was purchased from Sigma Chemical (St. Louis, MO, USA). Ammonium acetate (NH_4OAc), calcium chloride ($CaCl_2$), acetic acid (HOAc), water, and acetonitrile are of HPLC grade (Fisher Scientific, Springfield, NJ). Microcon centrifugal filter units YM-10 was obtained from Millipore (Bedford, MA, USA). *Escherichia coli* expression and purification of the recombinant *Flavobacterium heparinum* heparin lyase I, II, III (Enzyme Commission (EC) #s 4.2.2.7, 4.2.2.X, 4.2.2.8) were performed in our laboratory as previously described [27]. LMWHs were desalted by dialysis using 1-kDa molecular weight cut-off (MWCO) dialysis tube (Spectrum Laboratories, CA, USA) and lyophilized before nuclear magnetic resonance (NMR) analysis and re-dissolved in distilled water into stock solution (20 μg/μL) for liquid chromatography (LC)-mass spectrometry (MS) analysis. Digestion buffer (50 mM NH_4OAc, 2 mM $CaCl_2$, pH 7.0) was used for heparin lyase treatment.

2.2. Disaccharide analysis

Samples (100 μg) were added to 100 μL digestion buffer and mixed with heparin lyase I, II and III (20 mU each in Tris-HCl buffer, pH 7.0). Samples were sufficiently digested in a 37 °C water bath for 12 h. Removing the enzymes using a 10-kDa MWCO spin column terminated the enzymatic digestion. The filtrates were lyophilized and re-dissolved in distilled water at a concentration of 1 μg/μL. Reverse-phase ion-pairing liquid chromatography (RPIP–LC) with on-line electrospray ion-trap mass spectrometry (ESI–ITMS) analysis was performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Wilmington, DE, USA) equipped with a 6300 ion-trap and a binary pump [18].

2.3. Bottom-up analysis

Samples (100 μg) were added to 100 μL of digestion buffer and mixed with heparin lyase II (20 mU in Tris-HCl buffer, pH 7.0).