Abstract: Heparin has been used as a clinical anticoagulant for more than 50 years and it has been the second most used drug in the world. Unfractionated heparin are broke down by either specific lyase enzymes or chemical depolymerizations to obtain various of low molecular weight heparins (LMWHs) maintains much of the antithrombotic activity and provides a more predictable pharmacokinetic profile and a ease of use. Although this process is believed to be well regulated and inspected, LMWHs may still show distinct pharmacological and biochemical profiles primarily because of their structural differences. These differences which derived from LMWHs preparation process can be accurately and precisely identified by nuclear magnetic resonance (NMR) spectroscopy. This study is designed to compare the structural differences of 11 enoxaparin copies from different manufacturers. Using a combination of one- and two-dimensional high sensitivity NMR, we were able to accurately integrate the monosaccharide residues of LMWHs and exhibit the structural differences.

Keywords: Low Molecular Weight Heparins, Enoxaparin, Nuclear Magnetic Resonance Spectroscopy, Quantitative Analysis.

INTRODUCTION

Glycosaminoglycans (GAGs) are a linear, sulfated, negatively charged, specific group of polysaccharides composed of disaccharide repeating units consisting of uronic acid and amino sugars. GAGs can be divided into four classes based on the form of hexosamine, hexose or hexuronic acid that is present. These classes are 1) heparin and heparan sulfate (HS), 2) dermatan sulfate (DS) and chondroitin sulfate (CS), 3) hyaluronan, and 4) keratan sulfate (KS). GAGs are found on all animal cell surfaces in the extracellular matrix (ECM). They regulate a number of distinct proteins by binding them specifically, such as cytokines, morphogens, growth factors, and adhesion molecules on the cell surface and in the ECM [1]. GAGs play a significant role in cell signaling and development, angiogenesis, axonal growth, tumor progression, metastasis and anti-coagulation [2-4].

Heparin, most widely known GAG, is primarily utilized in pharmaceutical industry as an anticoagulant drug for the treatment of thrombosis, embolism, and thrombophlebitis. In 1980s, a unique pentasaccharide domain [GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S] was discovered in heparin which activates antithrombin III (AT), a serine protease inhibitor that blocks thrombin (factor IIa) and factor Xa in the coagulation cascade [5]. When AT binds to heparin, AT undergoes a conformational change which results in the inhibition of factor Xa and thrombin. AT binding pentasaccharide is sufficient to inactivate factor Xa. However, heparin chains with at least 16 saccharides are necessary to accelerate the reaction of AT with thrombin [6]. The identification of the 3-O-sulfo group as a marker for AT-binding site was an important achievement in the elucidation of the anticoagulant activity of heparin. This 3-O-sulfo group is absolutely necessary for the specific interaction of AT with heparin [7]. Pharmaceutical heparin is extracted from animal tissues, with the vast majority coming from porcine intestine. In 2008, nearly 100 people died due to the heparin they were administered, which was later found to have been contaminated with oversulfated chondroitin sulfate (OSCS) [8].
Low molecular weight heparins (LMWHs) are smaller chains of unfractionated heparin (UFH) which can be produced via diverse chemical and enzymatic depolymerization methods, for instance, while tinzaparin is produced by enzymatic depolymerization, enoxaparin (Lovenox, Sanofi-Aventis) is prepared by chemical β-eliminative reaction. LMWHs are more and more replaced with UFH due to theirs decreased hemorrhagic risk [9-11]. They have higher anti-Xa/anti-IIa activity ratios and thus their bioavailability is improved compared to UFH [12].

Nuclear magnetic resonance (NMR) spectroscopy technique has been widely used for characterization the structure of complex polysaccharides [13-15]. Evaluating and monitoring the structural differences among LMWHs from different producers is a vital step for development and approval of current and alternative anticoagulant drugs since most of these drugs are no longer protected by US patents.

In the current study, a comparison of eleven enoxaparin, the most widely used LMWH, samples from different manufacturers present within the same market was carried out by a combination of one- and two-dimensional quantitative NMR technique. This approach can provide an easy and more importantly dependable method for monitoring the similarities and differences among LMWHs.

**EXPERIMENTAL**

**Sample Preparation**

A 50 mg aliquot of each sample was prepared for NMR analysis by dissolving each in 0.5 mL of 99.996 atom% deuterium oxide (2H2O) and freeze dried to remove exchangeable protons. The 2H2O dissolution/freeze-drying was repeated three times before NMR analysis. After third 2H2O exchange, lyophilized powder samples were dissolved in 2H2O and transferred to a 5 mm NMR tube.

**Nuclear Magnetic Resonance (NMR) Analysis**

LMWH samples were analyzed by 1H-NMR and two-dimensional heteronuclear single quantum coherence (HSQC) NMR experiments to characterize their structures. LMWH samples were deuterium exchanged in 0.4 ml of 99.996% D2O to remove exchangeable protons. All NMR experiments were acquired on a Bruker Avance II Ultrashield 800 MHz (18.8-Tesla) NMR spectrometer with an ultrasensitive HCN cryoprobe. Probe temperatures of 298K, 313K, and 333K were used to determine better resolution for overlapping signals. 1D 1H experiments were acquired with 64 of number scans and acquisition time of 2.66 s. For two-dimensional NMR experiments, 128 experiments resulting in 4096 data points for a spectral width of 10 ppm were measured. Proton-detected HSQC experiments were measured using the ’hsqcgpplndqf’ pulse sequence and spectral widths of 12- and 78-ppm in the F2 (1H) and F1(13C) dimensions were used, respectively.

**Sparky for 2D Quantification**

The 2D spectra were quantified using the Sparky program (T.D. Goddard and D.G. Kneller, SPARKY 3, (USCF, http://www.cgl.ucsf.edu/ home/sparky). In order to get reproducible results, following settings were used: Integration method, Gaussian fit; fit baseline, yes; use data above lowest contour, no; use data within rectangle, yes; maximum motion was set to 0.01, line width minimum and maximum (Hz) were 2.0 and 80.0; maximum minimization steps equal to 10,000 and minimization tolerance was 0.1%. After necessary settings were made, peaks to be integrated was selected by “find/add peak” in the pointer mode box. Next, we define a box around the peaks to be integrated and the regions within the box that are not defined as peaks were employed to determine the baseline. A non-zero baseline correction was applied.

**RESULTS AND DISCUSSION**

Heparin is a linear and mostly sulfated polysaccharide composed of the major disaccharide unit β-1,4-linked hexuronic acid (HexA) and glucosamine (GlcN). It is the second most used drug (after insulin) that is currently used as an anticoagulant drug during very important health operations including open heart surgery, stroke and dialysis bags.

Anticoagulant activity of heparin arises mainly through a well-defined unique pentasaccharide sequence that specifically binds to antithrombin III which inhibits blood coagulation proteins including thrombin and factor Xa. LMWHs are the most commonly prescribed anticoagulants (34 % of the patients). Tinzaparin, dalteparin and enoxaparin can be regarded as top three utilized LMWHs. Their preparation processes from UFH differ from each other.
While tinzaparin is produced by enzymatic depolymerization, dalteparin is produced by nitrous acid depolymerization. Enoxaparin (Lovenox), the most used LMWH drug, is produced by benzylation followed by alkaline depolymerization (Figure 1).

![Scheme of depolymerization used to prepare low molecular weight heparins (Enoxaparin, Tinzaparin and Dalteparin). Major reducing and non-reducing end residues are of LMWHs are shown as well as antithrombin-pentasaccharide binding site structure.](image)

Figure 1: Scheme of depolymerization used to prepare low molecular weight heparins (Enoxaparin, Tinzaparin and Dalteparin). Major reducing and non-reducing end residues are of LMWHs are shown as well as antithrombin-pentasaccharide binding site structure.

![1H NMR spectrum of Enoxaparin no. 1. Major signals are identified. The spectra were acquired at 600 MHz (14.1-Tesla) on a Bruker Avance Ultrasound NMR instrument.](image)

Figure 2: $^1$H NMR spectrum of Enoxaparin no. 1. Major signals are identified. The spectra were acquired at 600 MHz (14.1-Tesla) on a Bruker Avance Ultrasound NMR instrument.
Figure 2 shows the $^1$H NMR spectrum of enoxaparin sample no 1. Major signals in $^1$H NMR spectrum correspond to major trisulfated disaccharide unit: $\text{4}^{-\text{α-L-IdoA}}_{\text{2SO}_3} \text{–α-(1→4)} \text{–D-GlcNSO}_3,\text{6SO}_3 \text{(I}_2\text{S–ANS}_6\text{S)}$. All proton resonances are found between 2 and 6 ppm for all LMWH samples in $^1$H-NMR. Since enoxaparin is produced by chemical β-eliminative reaction, it possesses 4-5 unsaturated 2-0-sulfated uronic acid (ΔU2S) at the non-reducing end of the polymer chain that can easily be recognized by a signal around 5.95 ppm. In addition, a small and unique peak at 5.85 ppm corresponding to H4 of unsaturated nonsulfateduronic acid (ΔU) are a specific signal for enoxaparin compared to other LMWHs. Most of the main $^1$H NMR signals in enoxaparin belongs to the original heparin structure and were readily determined by previous studies [16-18].

$^1$H NMR analysis of enoxaparin samples was acquired at 3 different probe temperatures (298K, 313K, and 333K). As it can be seen from Figure 3a, anomeric signals of nonsulfatediduranic acid (I) peak is overlapped with HDO solvent peak. Thus, it was very unlikely to obtain correct integration values for (I) signal. We have tried three different probe temperatures in order to get more reliable integration results for in particular anomeric signals. Since the NMR instrument is locked on the solvent signal (deuterated water, HDO) around 4.9 ppm, peaks other than the solvent’s peak will shift to the downfield of the spectrum with the increase of the probe temperature (Figure 3). The best resolution of anomeric signals was achieved at a probe temperature of 313 K.

![NMR Spectrum of Enoxaparin](image)

**Figure 3:** $^1$H NMR spectrum of Enoxaparin no. 1 in three different probe temperatures: 298 K (a), 313 K (b), 333 K (c).

Although nuclear magnetic resonance (NMR) spectroscopy is an expensive and mass-limited technique, it can be regarded as one of the most essential structural elucidation method for complex glycosaminoglycan analysis. While mono-dimensional (1D) NMR spectroscopy provides information about chemical shift values, integration, J-coupling constants and samples’ cleanness, in some cases, two-dimensional (2D) NMR experiments are required for further analysis of LMWHs. Severe overlapping of the peaks (3-5 ppm) in the mono-dimensional NMR spectra of LMWHs prevents their use for quantitative analysis. Thus, 2D NMR techniques should be used to overcome the overlapping issue which might cause unreliable integration namely quantification results.
The anomeric and ring regions of 2D $^1$H-$^{13}$C NMR heteronuclear multiple quantum coherence (2D-HSQC) spectrum of enoxaparin is shown in Figure 4. All the main peaks were assigned based on the previous NMR studies[19] and signals that were employed for monosaccharide compositional analysis were underlined. Two signals that are specific to enoxaparin samples compared to other LMWHs are 2-amino-1,6-anhydro-2-deoxy-$\beta$-D-mannopyranose (1,6-an.M) and 2-amino-1,6-anhydro-2-deoxy-$\beta$-D-glucopyranose (1,6-an.A). These two unique bicyclic structures present at the reducing end originating from alkaline treatment of UFH. Another very important signal that cannot be integrated due to severe overlapping in 1D $^1$H NMR spectrum is that glucuronic acid linked to ANS3S (G-ANS3S) residue. G-ANS3S has only been detected in the unique pentasaccharide residue can readily be resolved and integrated at 4.6/102 ppm in 2D HSQC spectrum.

Figure 4: Two-dimensional $^1$H-$^{13}$C NMR correlation spectrum of two-dimensional heteronuclear single quantum coherence (2D-HSQC) of Enoxaparin no. 1. a) anomeric region of 2D-HSQC spectra of enoxaparin and b) ring signals of 2D-HSQC spectra of enoxaparin.

The comparison of three different brands enoxaparin was carried out to compare their monosaccharide composition by 2D-HSQC experiments since enoxaparin is the most commonly used LMWH (Table 1). Enoxaparin samples essentially show very similar monosaccharide composition as we expected from their 1H NMR spectra. Nonetheless, enox-1 has the highest amount of glucuronic acid linked to ANS3S (G-ANS3S) which is the unique disaccharide sequence has only been detected in AT binding pentasaccharide. While enox-3 sample showed higher GlcA, IdoA and Glc6S levels than other enoxaparin samples, enox-1 demonstrated higher IdoA2S and most importantly ANS3S amount (Figure 5). In addition, degree of sulfation, calculated by adding all sulfated monosaccharides, was quite similar for all enoxaparin samples (2.5-2.6).
Figure 5: Comparison of the monosaccharide compositions of three enoxaparin samples by NMR integration.

Table 1: Monosaccharide composition of LMWHs by two dimensional HSQC.

| LMWH | A₈s | Aₐc | G‐ | A₈₅ | Aₐₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙₙ$_{1,6}$-an. A, 2-amino-1,6-anhydro-2-deoxy-$\beta$-D-glucopyranose; 1,6-an. M, 2-amino-1,6-anhydro-2-deoxy-$\beta$-D-mannopyranose

CONCLUSIONS

Enoxaparin is the most widely used LMWH that is produced by the depolymerization of unfractionated heparin.

Since LMWHs can be produced by various manufacturing processes, it is very important to monitor their structural similarity or differences.

Using a combination of one- and two-dimensional NMR experiments, we analyzed structural peculiarities of three different brands enoxaparin samples. Although extended and further researches are needed to utterly perceive the structure and biological features of these drugs, current study provides such significant information including monosaccharide compositions, quantification of components, any possible impurities if present and degree of sulfation.

CONFLICT OF INTEREST

The author stated that did not have a conflict of interests.
REFERENCES


