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# Molecular weight of low molecular weight heparins by $^{13}C$ nuclear magnetic resonance spectroscopy

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ABSTRACT

Heparin and low molecular weight heparins are polydisperse polysaccharides with a degree of polymerization ranging from 4 to ~40. The determination of their average molecular weights has traditionally relied on size exclusion chromatography involving the use of oligosaccharides of known size and molecular weight as standards. $^{13}C$ NMR spectroscopy is applied for the first time to obtain the molecular weights of low molecular weight heparins. The signal intensities of the reducing end and internal anomeric carbons, having distinctive chemical shifts in the $^{13}C$ NMR spectrum, are measured to determine the molecular weight. Compared to techniques utilizing broad band decoupling or selective decoupling of anomeric protons, distortionless enhancement polarization transfer pulse sequence gave better quantitation of signal intensities of anomeric carbons. Molecular weight was calculated from the calibrated ratio of signal intensities of the anomeric carbons of reducing end groups and internal residues, and the disaccharide compositional analysis. The calibrated signal intensity ratio is determined using the $T_1$ relaxation rates of anomeric carbons of model oligosaccharides. The disaccharide composition of low molecular weight-heparins is obtained using capillary electrophoresis. Signal averaging over 40000–90000 transients, requiring a total of 12–18 h on a 360-MHz NMR spectrometer was adequate to measure molecular weights in the range of 3000–7000. The measured molecular weights of twelve low molecular weight heparins, analyzed by this $^{13}C$ NMR spectroscopic technique, correlated well with the number average molecular weights obtained using high performance-gel permeation chromatography and gradient polyacrylamide gel electrophoresis. In addition to establishing the number average molecular weight, the $^{13}C$ NMR spectra helped distinguish the structural properties of different commercially prepared low molecular weight heparins.

## INTRODUCTION **

Heparin is a highly sulfated, polydisperse, alternating copolymer of (1 → 4)-linked 2-deoxy-2-amido-glucopyranose and pyranosyluronic acid residues. The

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** Abbreviations used: LMW, low molecular weight; FID, free induction decay; DEPT, distortionless enhancement polarization transfer; BB, broad band; TSP, 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid, sodium salt; GPC, gel permeation chromatography; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; dp, degree of polymerization; $t_r$, retention time; $d_m$, migration distance; AU, absorbance units; $V_t$, total volume; $V_0$, void volume; $P$, polydispersity; CE, capillary electrophoresis; MWCO, molecular weight cut-off; INEPT, insensitive nuclei enhanced by polarization transfer.


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major repeating structural unit of heparin polysaccharide is the trisulfated disaccharide sequence 4)-*O*-(2-deoxy-2-sulfoamino-α-D-glucopyranosyl)-(1 → 4)-(α-L-idopyranosyluronic acid 2-sulfate)-(1 → . Although this sequence constitutes ~ 60–75% of the polysaccharide chain in heparins from different sources[1], considerable structural and sequence variability makes the precise structure of heparin difficult to determine.

The principal clinical use of heparin over the past 5 decades has been as an anticoagulant[2]. About 500 million doses a year are used worldwide in variety of surgical procedures, yet heparin has been cited as the principal cause of death in otherwise healthy patients[3]. Attention has been focused on low molecular weight (LMW) heparins as they have been claimed to exhibit reduced side effects, more predictable pharmacological action, together with retaining high in vivo antithrombotic activity[4–6]. Low molecular weight heparins are also claimed to possess better bioavailability[7]. These potential advantages have led to the development of many commercial LMW-heparin preparations. The different methods used in the preparation of these LMW-heparins, together with the polydispersity of the heparins from which they are derived, ensures high structural variability and polydispersity in LMW-heparins. The anticoagulant activity of LMW-heparins has been found to be dictated not only by the presence of a unique antithrombin binding site but also by the length of the polysaccharide chain[8]. However, the reduction in heparin's molecular weight reduces the anticoagulant effect, associated with bleeding complications, to a greater degree than the desired antithrombotic effect[4,9,10]. Thus, the determination of the molecular weight of structurally diverse LMW-heparins is very important.

Numerous methods have been explored for determining the molecular weights of heparins and LMW-heparins. Viscometric determination of the molecular weight of heparin relies on the Mark–Houwink equations[11,12]. Ultracentrifugation uses the Svedberg equation to determine the molecular weight of heparin[11,13], however, its dependence on the partial specific volume of heparin (a term that is difficult to measure) can lead to substantial error in the measured molecular weight[14]. Light scattering methods[13], including quasielastic light scattering[15], and low angle X-ray scattering[16], have also been used. Heparin is a relatively small biopolymer making analysis by light scattering techniques difficult. These problems are compounded when low molecular weight heparins are analyzed.

Gel permeation chromatogray (GPC)–HPLC is a low resolution technique, typically used for determining number average ($M_n$) and weight average ($M_w$) molecular weight of heparins and LMW-heparins[17]. It relies on ultraviolet absorbance detection at 206 nm, which is not selective for LMW-heparin, and nonheparin contaminants can interfere with the molecular weight determination. The low extinction coefficient of the carboxylate chromophore of LMW-heparin requires a high sample concentration and may result in interchain interactions leading to inaccurate molecular weight $M_r$ values.

Recently gradient polyacrylamide gel electrophoresis (PAGE) has been introduced as a high resolution technique for the determination of the molecular weight of heparins[18]. However, this technique also has limitations. The relationship between staining intensities and mass for higher oligosaccharide (above dp 8) becomes semilogarithmic when intense silver staining is used to improve sensitivity[29]. In addition, the staining of polysaccharide chains of heparin is proportional to the number of sulfate groups on the chain. Thus less sulfated oligosaccharides, are stained to a lesser extent than highly sulfated oligosaccharides, of the same size.

Both GPC–HPLC and gradient PAGE need defined oligosaccharide molecular weight standards for accurate calibration. The preparation of such defined oligosaccharide standards (dp 6 to ~20), by controlled depolymerization of heparin followed by high resolution fractionation, purification, and characterization, would require an enormous effort.

Although $^{13}C$ NMR spectroscopy has been used extensively in the structure elucidation of heparin and heparin derivatives[19–23], the applicability of this technique to the determination of average molecular weights of heparins or LMW-heparins has not been well studied. Casu et al.[19] reported the use of proton-noise decoupled $^{13}C$ NMR spectroscopy for estimation of the degree of polymerization of enriched fractions of oligosaccharides. However, this analysis of very low molecular weight oligosaccharides does not take into account the differential relaxation of the signals involved. We present the first use of $^{13}C$ NMR spectroscopy to obtain a standard-free measure of the average molecular weight of LMW-heparin samples.

## EXPERIMENTAL

*Materials.*—The LMW-heparin samples used in this study were a gift from Dr. J. Fareed, Loyola University Medical Center, Maywood, IL [Enoxaparin (PK10169), Logiparin (LHN-1), Fraxiparin (CY216), Fluxum (OP2123), and Fragmin (KABI2165)] or were multiple lots obtained from Wyeth-Ayerst Research Laboratories, Rouses Point, NY (RD heparins a–g). Bovine lung heparin, blue dextran ($M_r$ 2 000 000), and SP-Sephadex C-50 were from Sigma Chemical Co. (St. Louis, MO). Heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (heparitinase II, no assigned EC number), and heparin lyase III (heparitinase I, EC 4.2.2.8) were prepared by hepąrin induced fermentation of soil bacterium *Flavobacterium heparinum* (also known as *Cytophaga heparina*) in our laboratory and purified to homogeneity[24]. Heparin lyase I had an activity of 130 IU/mg against heparin, heparin lyase II had an activity of 19 IU/mg and 36.5 IU/mg against heparin and heparan sulfate, respectively, and heparin lyase III had 63.5 IU/mg activity against heparan sulfate. Acrylamide (electrophoresis grade), tris(hydroxymethyl)amino methane (Tris), Alcian Blue dye, bromophenol blue dye, and ammonium persulfate were obtained from Boehringer Mannheim (Indianapolis, IN). Sodium azide, glycine hydrochloride, disodium ethylenediaminetetraacetic acid, Azure A dye,

boric acid, sucrose, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Fischer Chemical Company (Fair Lawn, NJ). Deuterium oxide ($^2H_2O$, 99.996% atom $^2H$) and TSP were from Aldrich Chemical Company (Milwaukee, WI). Sodium sulfate (anhydrous) was from EM Science (Gibbstown, NJ). Spectropore dialysis membranes (MWCO 1000 and 3500) were from Spectrum Medical (Los Angeles, CA).

*Instrumentation.*—$^{13}C$ NMR experimentation was performed on WM 360 NMR spectrometer from Bruker Spectrospin (Switzerland). GPC–HPLC was performed using a TSK 3000SW and a TSK 2000SW (30 × 0.75 cm) column in series. A model III LDC Constametric pump (Riveira Beach, FL) and a 2141 LKB Bromma, variable wavelength detector (Sweden) were used for HPLC. Gradient PAGE was performed on a Hoefer (San Francisco, CA) SE600 vertical slab-gel unit equipped with a Bio-Rad model 1420B power source. The densitometric scanning of stained gels was performed using an image grabber software on a Macintosh II computer. The image was recorded with computer controlled Kodak Megaplus online video camera and digitized by means of Pixels tools software from Perceptics. Capillary electrophoresis was performed using a Dionex Capillary Electrophoresis System with Advanced Computer Interface, Model I, equipped with high voltage power supply capable of constant or gradient voltage control from Dionex Corporation (Sunnyvale, CA). The uncoated silica capillaries (75 $\mu$m internal diameter, 64 cm effective length) were from Dionex. Measurements of pH were obtained using a Beckmann $\Phi$40 pH meter.

*Preparation of LMW-heparins for $^{13}C$ NMR.*—LMW-heparins (~3 g) were dissolved in 6 mL deionized, distilled water and the pH of the solution was adjusted to 7.0. The solution was filtered through 0.45-$\mu$m membrane and freeze-dried. The resulting solid was reconstituted in 3 mL $^2H_2O$ (99.9% atom $^2H$) containing 0.03% (w/v) TSP, and freeze-dried. This process was further repeated twice with 3 mL portions of $^2H_2O$ (99.996% atom $^2H$). The final solution was degassed overnight under vacuum and stored at −20°C.

*$^{13}C$ NMR Spectroscopy of LMW-heparins.*—The proton noise decoupled $^{13}C$ NMR spectra of LMW-heparins were recorded using 16K data points, with an acquisition time of 0.344 s and spectral width of 23809 Hz. The decoupler frequency was set at the center of anomeric proton multiplet (6826 Hz) with 2 W decoupler power to ensure equal NOE effects on all anomeric carbons. A relaxation delay of 0.66 s was used. Typically 40 000–90 000 fid's were acquired over an 8–16 h period. The fid's were Fourier transformed using a 1.0–3.0 Hz line broadening factor for higher signal–noise ratio.

The DEPT spectra were recorded using the standard Bruker software in DISN85 version. The polarization transfer was optimized for observing only tertiary carbons using $^1J_{CH}$ 170 Hz for anomeric carbons[25]. A recovery delay of 0.66 s was used. The low power decoupler frequency was set in the center of anomeric proton multiplet (6826 Hz) with 0.4 W decoupling power. Typically 20 000–60 000 fid's were acquired, each with 16K data points, 0.34 s acquisition

time, 23809 Hz spectral width, and Fourier transformed using an exponential multiplication factor of 1.0–3.0 Hz.

The broad band decoupled and DEPT spectra were recorded at 298 K as well as 323 K to observe the effect of higher temperature on relaxation times of anomeric carbons. No significant improvement in the signal intensity of anomeric carbons of reducing end residues was observed at 323 K. Thus all further measurements were performed at 298 K. The $^{13}C$ NMR spectra were referenced by setting the methyl carbon signal of TSP to 0.0 ppm.

*$T_1$ Relaxation studies.*—To obtain the relaxation times of anomeric carbons for quantitation of carbons signals, a tetrasaccharide was chosen. This tetrasaccharide[21] **1** (Fig. 1) is constituted of 2-*O*-sulfated idopyranosyluronic acid (unit b) and 2-deoxy-2-sulfoamino-glucopyranosyl 6-sulfate (unit c) residues, which represent the most prevalent monosaccharide units in heparin and consequently would be expected to reflect the relaxation times of anomeric carbons of heparin. $T_1$ Relaxation studies for anomeric protons were also performed on tetrasaccharide **1** and hexasaccharide[26] **2** (Fig. 1) as these represent the various functionalizations possible on the heparin chain.

The longitudinal relaxation times of anomeric carbons were obtained using an inversion recovery pulse sequence. Approximately 150 mg of tetrasaccharide **1** was dissolved in 500 $\mu$L $^2H_2O$ (99.9% atom $^2H$) containing TSP and lyophilized. This process was repeated twice with 500 $\mu$L $^2H_2O$ (99.996% atom $^2H$) at room temperature. The variable delays used for the inversion recovery pulse sequence were: 5 ms, 10 ms, 20 ms, 50 ms, 80 ms, 100 ms, 150 ms, 200 ms, 300 ms, 500 ms, 800 ms, 1 s, 2 s, 5 s, and 10 s. The processing was performed on ASPECT 2000 terminal using Bruker software. The longitudinal relaxation rates of anomeric protons of tetrasaccharide **1** and hexasaccharide **2** were also obtained using an inversion recovery pulse sequence under similar acquisition.

*Two-dimensional NMR analysis of Logiparin.*—The heteronuclear ($^{13}C$–$^1H$) correlation spectroscopy was performed on Logiparin (LHN-1) using standard Bruker software on a WM-360 NMR instrument at an operating frequency of 90.56 MHz for the $^{13}C$ nucleus. A total of 128 data points were acquired in the $T_1$ dimension each composed of 320 scans. The fid's were Fourier transformed onto a data matrix of 1024 × 512W in absolute intensity mode and plotted as contours. The magnetization transfer was optimized for a coupling constant $^1J_{CH}$ of 135 Hz and a relaxation delay of 2.0 s was used. The homonuclear ($^1H$–$^1H$) correlation spectroscopy was performed on Logiparin (LHN-1) using a d1–$\pi$/2–d2–$\pi$/2–fid pulse program in the absolute intensity mode. The acquisition and processing parameters were similar to a HETCOR experiment.

*Preparation of LMW-heparin samples.*—Stock solutions of LMW-heparins for GPC-HPLC, gradient PAGE, and CE analysis were prepared by dissolving the bulk drug at ~ 20 mg/mL concentration in deionized, distilled water and filtering through a 0.45-$\mu$m membrane. The pH of the solution was adjusted to 7.0 with 0.1

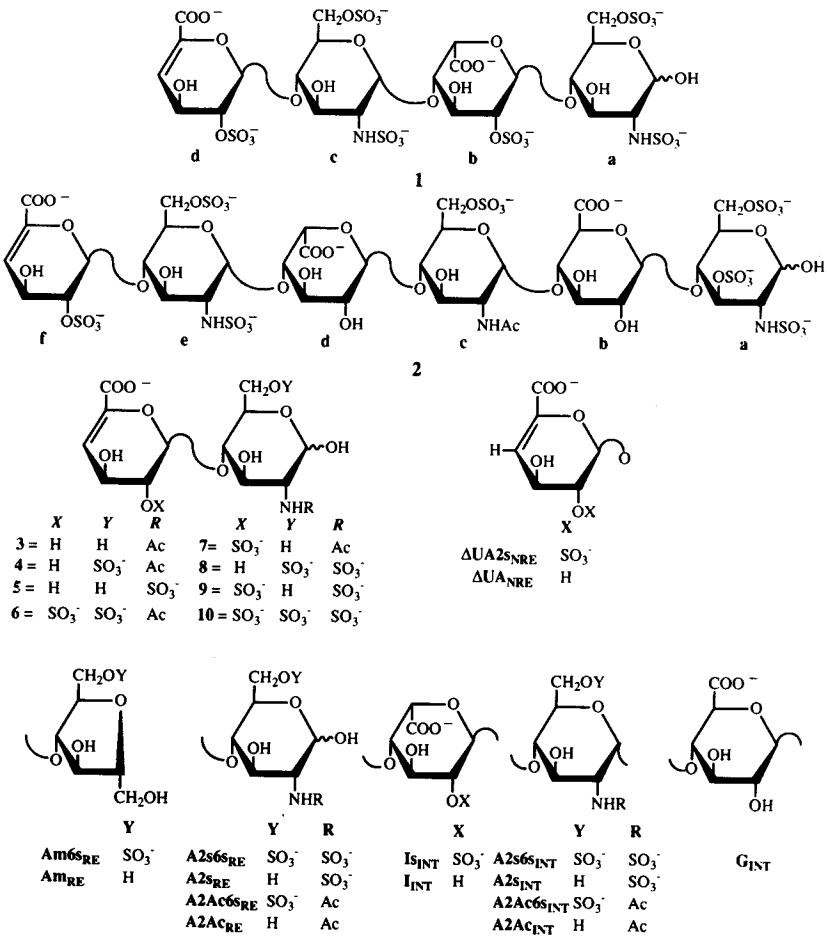


Fig. 1. Structure of tetrasaccharide **1**, hexasaccharide **2** and disaccharides **3–10**. Saccharide residues found in LMW-heparins are shown where X, Y, and R designate structural variability. The reducing end, nonreducing end, and internal saccharide units are designated with RE, NRE, and INT subscript, respectively.

N NaOH. The filtrate was freeze-dried and reconstituted to an exact concentration of 20 mg/mL with deionized, distilled water.

*Preparation of a standard oligosaccharide mixture for GPC–HPLC and gradient PAGE.*—For the purpose of generating a calibration curve of molecular weight values with retention time ($t_R$) in GPC–HPLC and with migration distance ($d_m$) in gradient PAGE, partial enzymatic digestion of bovine lung heparin was performed. Bovine lung heparin (1 g) was dissolved in 15 mL of 5 mM sodium phosphate buffer (pH 7.1) containing 100 mM NaCl and filtered through 0.45-$\mu$m membrane. The solution was brought to 30°C and a 4-$\mu$L aliquot withdrawn for measuring the absorbance of the control sample. Heparin lyase I (100 mU) in 5 mM sodium phosphate buffer (pH 7.1, 20 $\mu$L) containing 100 mM NaCl was then added and

the absorbance of the digestion mixture at 232 nm was monitored periodically by withdrawing a 4-$\mu$L aliquot and diluting to 750 $\mu$L with 30 mM HCl. After a 12-h digestion period, an additional 100 mU of heparin lyase I was added. When the final UV absorbance reached 0.2 AU ($\sim$ 20% digestion), the digestion mixture was reduced to pH 2.5 with 1 N HCl and passed through an SP-Sephadex C-50 column (25 mL, 1 g resin in 65 mL water at pH 2.0) to remove the heparin lyase I. The solution was neutralized to pH 7.0 with 1 N NaOH. To remove salt and low molecular weight (dp < 8) oligosaccharides, the solution was dialyzed against 2 × 1000 vol of deionized, distilled water with MWCO 3500 dialysis membrane at 4°C over a 12 h period. The resulting solution was freeze-dried and reconstituted to an exact concentration of 20 mg/mL with deionized, distilled water.

*Molecular weight analysis of LMW-heparins by GPC–HPLC.*—Two columns, TSK 3000SW and 2000SW (30 × 0.75 cm, 10-$\mu$m particle size), were connected in series and equilibrated with 250 mM sodium sulfate (pH 4.5). Blue dextran ($M_r$ 2 000 000) and sodium azide were used to obtain the $V_0$ and $V_t$ values respectively. Prior to injection of LMW-heparins, the GPC system was calibrated using the partially depolymerized bovine lung heparin which served as molecular weight standards. A flow rate of 0.6 mL/min was used and the eluent was monitored at 232 nm. Identification of the peaks was carried out using well characterized oligosaccharides[21,27,28] of dp 2, 4, 6, and 8. A calibration curve was constructed by third order polynomial regression analysis with coefficients $C_0$, $C_1$, $C_2$, and $C_3$. The coefficient of variation was 0.998. LMW-heparin (15 $\mu$L, 20 mg/mL) was then analyzed at a flow rate of 0.6 mL/min and the eluent was monitored at 206 nm. The resulting chromatogram was sliced into approximately 200 time slices providing 200 $M_r$ and $t_R$ values. Values for retention time ($T_R$) and the corresponding absorbance values were then imported into a personal computer spreadsheet program. The molecular weight corresponding to each $t_r$ value was calculated from the calibration curve using eq *1*.

$$\log(M_r) = C_0 + C_1(t_r) + C_2(t_r)^2 + C_3(t_r)^3 \quad (1)$$

The 200 $M_r$ values so obtained were transformed into $M_n$, $M_w$, and $P$ values using the absorbance values as given by eqs 2, 3, and 4.

$$M_n = \sum A_i M_i / \sum A_i \quad (2)$$

$$M_w = \sum A_i M_i^2 / \sum A_i M_i \quad (3)$$

$$P = M_w / M_n \quad (4)$$

*Molecular weight analysis of LMW-heparins by gradient PAGE.*—The preparation of gradient polyacrylamide gels was carried out as reported earlier[10,18]. Briefly, a gradient of 11.5% (w/v) acrylamide to 20.2% (w/v) acrylamide of 1.5-mm thickness was prepared between glass plates (20 × 22 cm). A comb was inserted during the polymerization process to form wells for loading samples of LMW-heparins.

Each well was rinsed with electrophoresis buffer. LMW-heparin (5 $\mu$L, 20 mg/mL) was combined with 50% (w/v) sucrose in distilled water (5 $\mu$L) and loaded onto the bottom of each well with a microsyringe. Each gel had a lane of standard oligosaccharide mixture obtained from partial digestion of bovine lung heparin for calibration. Electrophoresis was performed for 8 h at 400 V while cooling the system using circulating tap water at 10°C. The gel was removed from the glass plates, stained with Alcian Blue (0.5%, w/v) in 2% (w/v) aq acetic acid for 15 min. Excess Alcian Blue dye was removed by repeated washings over a 2 day period with deionized, distilled water. The Alcian Blue stained gel was then lightly silver stained for better clarity and higher sensitivity as previously described[29].

For the purpose of determining the $M_r$ values of LMW-heparins, the stained gel was digitized by an online computer controlled video camera. The scan frame was adjusted for the image of the entire gel using autocontrolled shutter speed and manually controlled focusing. The resolution of the image of the gel was set at $1312 \times 1024$ pixels. The image so obtained was then compressed to a 300 to 500K file size for processing in a personal computer. Each lane was digitized using a digitizing program to generate a plot of particle density versus distance in pixels. A calibration curve was constructed using partially depolymerized bovine lung heparin. Band assignment was performed using well characterized oligosaccharides[22,24] of dp 2, 4, and 6. A good correlation between log ($M_r$) values and distance in pixels ($d_m$) was observed using third-order polynomial regression analysis as given in eq 5.

$$\log (M_r) = D_0 + D_1(d_m) + D_2(d_m)^2 + D_3(d_m)^3 \quad (5)$$

The coefficient of variation was found to be 0.97. The values for $M_n$, $M_w$, and $P$ were then calculated in a manner similar to that described for GPC–HPLC analysis.

*Complete enzymatic depolymerization of LMW-heparin.*—Heparin lyase I, heparin lyase II, and heparin lyase III were each added (25 mU/mg of substrate) to a solution of LMW-heparin sample in 5 mM sodium phosphate buffer (500 $\mu$L/mg of substrate) at pH 7.0 containing 100 mM NaCl. The enzymatic depolymerization proceeded for 12 h at a controlled temperature of 30°C. A time of 12 h was found sufficient for the complete depolymerization of LMW-heparins as monitored by no increase in absorbance at 232 nm. The depolymerized samples were freeze-dried and reconstituted in deionized, distilled water at a concentration of 2 $\mu$g/$\mu$L.

*CE analysis of LMW-heparin.*—Separation and analysis of the completely depolymerized LMW-heparins were carried out using a fused silica capillary column (75-$\mu$m internal diameter, 64 cm effective length). A new capillary was activated by extensively washing with 0.1 M phosphoric acid, 0.5 M NaOH, distilled, deionized water, and run buffer (see Table II legend) before use. The sample was injected using hydrostatic pressure to give 14.6 nL total volume of injected solution. The electrophoresis was performed using 20130 V constant voltage. The detection system consisted of a variable-wavelength ultraviolet detector operating at 232 nm.

The disaccharide peaks were identified by injecting a mixture of eight well characterized disaccharides **3–10** (Fig. 1). For disaccharide compositional analysis[30], a baseline was set at the beginning and at the end of each recognizable peak.

## RESULTS AND DISCUSSION

LMW-heparin can be prepared in low yields (< 15%) by size fractionation using size exclusion chromatography from heparin. Controlled, partial depolymerization of heparin gives much higher recoveries of LMW-heparin. Chemical or enzymatic depolymerization may, however lead to modification of basic structural characteristics of heparin introducing artifacts that may alter the biological properties of a LMW-heparin[10,31].

The numerous methods of preparing LMW-heparins result in bulk drugs with wide differences in biochemical and pharmacological properties[10]. One property that is affected the most is the molecular weight. Although numerous techniques have been investigated for determining the molecular weight, the unavailability of structurally defined oligosaccharide standards poses a severe problem. The application of $^{13}C$ NMR spectroscopy to determine molecular weight, could potentially overcome the problems associated with the unavailability of standards. An additional asset of this method is its usefulness in identifying and quantitating a variety of structural features present in LMW-heparins. $^{13}C$ NMR spectroscopy has been investigated for the structural analysis of heparin and heparin-derived oligosaccharides[19–21,23,32]. Quantitative $^{13}C$ NMR spectroscopy, however, requires a knowledge of structure as well as the longitudinal relaxation times and NOE effects of the carbons being studied[33].

*Relaxation studies.*—To optimize the relaxation delay for measuring $^{13}C$ NMR spectrum, the proton longitudinal relaxation times of anomeric protons were first measured using an inversion recovery pulse sequence on tetrasaccharide **1** and hexasaccharide **2** (Table I). The longitudinal relaxation times of glucosamine residues were found to be higher than those for uronic acid residues suggesting greater flexibility and mobility of uronic acid residues. The anomeric proton of unsaturated uronic acid residues relaxes relatively slow. These residues, however, do not constitute a major proportion of the polysaccharide chain of most LMW-heparins and hence, need not be considered. All other anomeric protons, irrespective of their nature or adjacent functionalizations, relax within 1 s suggesting an optimal repitition rate of about the same.

The $T_1$ relaxation times for anomeric carbons of internal $\alpha$-L-idopyranosyluronic acid and 2-deoxy-2-sulfoamino-$\alpha$-D-glucopyranosyl 6-sulfate residues, and the reducing end residue were found to be significantly different (Table I). While the internal uronic acid residue and glucosamine residue had an average longitudinal relaxation time of 241 ms, the reducing end 2-deoxy-2-sulfamido-$\alpha$-D-glucopyranosyl 6-sulfate residue had a $T_1$ value of 220 ms. The longitudinal relaxation rates of anomeric methine carbons indicates that although the molecular mass of

TABLE I

Longitudinal (spin–lattice) relaxation times for anomeric carbons and protons of heparin oligosaccharides **1** and **2** at 298 K using an inversion-recovery pulse sequence

| Residue [a] | $T_1$ (s) ± sd | | |
|---|---|---|---|
| | Anomeric carbon | Anomeric proton | |
| | **1** | **1** | **2** |
| a | 0.220 ± 0.050 | 1.023 ± 0.006 | 0.932 ± 0.014 |
| b | 0.267 ± 0.037 | 0.727 ± 0.002 | nd [b] |
| c | 0.215 ± 0.071 | 0.823 ± 0.024 | 0.971 ± 0.015 |
| d | 0.354 ± 0.062 | 0.774 ± 0.012 | 0.720 ± 0.162 |
| e | | | 0.890 ± 0.057 |
| f | | | 2.037 ± 0.094 |

[a] See Figure 1. [b] Not determined due to overlapping signals.

the highly anionic tetrasaccharide **1** is less than 1000 Da, the rate of molecular tumbling is slow. For LMW-heparins, having a molecular weight of ~3000–8000 Da, the slow molecular tumbling is expected to hold true. Therefore, $T_1$ values of anomeric methine carbons of tetrasaccharide were used for quantitating the anomeric CH signal intensities of LMW-heparins. The $T_1$ relaxation time for α-L-idopyranosyluronic acid 2-sulfate is higher than for 2-deoxy-2-sulfoamino-α-D-glucopyranose 6-sulfate, indicating greater conformational freedom and local movement for uronic acid residues. This conformational flexibility has also been observed for oligosaccharides[34] by $^1$H NMR spectroscopy.

The signal intensity ($S$) of a nucleus (i) at any time $\tau$ is related to its relaxation rate ($T_{1i}$) by eq *6*.

$$S_i(\tau) = S_i(0)\ e^{-\tau/T_{1i}} \quad (6)$$

where $S_1(0)$ represents the signal intensity of nucleus i at time 0. Rearranging eq *6* for internal and reducing end anomeric carbons and substituting with an acquisition time of 0.344 s yields eq *7* (and eq *8* for unsaturated nonreducing end residue containing LMW-heparins).

$$S_{int}(\tau)/S_{red}(\tau) = 1.15 \quad (7)$$

$$S_{int,nre}(\tau)/S_{red}(\tau) = 1.39 \quad (8)$$

The signal intensity of internal anomeric carbons are calculated to be greater than the signal intensities of the reducing end anomeric carbons by a factor of 1.15. Therefore, quantitative $^{13}$C NMR requires that the intensity of the reducing end anomeric carbons be multiplied by a factor of 1.15 before being compared to the internal anomeric carbons.

*Nuclear Overhauser effect.*—Although nuclear Overhauser enhancement can be due to number of different relaxation mechanisms, heteronuclear dipolar relaxation is the dominant effect in $^{13}$C NMR spectroscopy. The NOE factor ($\eta$) for

such a relaxation mechanism is governed to a large extent by the gyromagnetic ratios ($\gamma$) and can be approximated by eq 9 (ref 33).

$$\eta = 1 + \gamma_C/2\gamma_H \quad (9)$$

Differences in the NOEs of carbons, principally arises due to the primary, secondary, or tertiary nature of the carbons involved. In addition, differences in NOEs may also occur due to differences in the rotational correlation times. For anomeric carbons, the rotational correlation times for internal and end residues are expected to be different, although the differences would be small. Thus, when quantitating the anomeric carbons in LMW-heparins, the NOE factor is assumed to be the same for all anomeric carbons as long as the proton decoupling channel is applied for a sufficiently long period and power to effect complete proton population inversion.

*$^{13}C$ NMR spectra.*—The assignment of the reducing end anomeric carbons was confirmed by using two dimensional heteronuclear ($^{13}C$–$^{1}H$) correlation spectroscopy for Logiparin. A distinctive signal at 93 ppm was found to correlate with anomeric proton at $\delta$ 5.4. This indicates that reducing end anomeric carbons of LMW-heparin occur between 92–96 ppm. A group of peaks between 99–105 ppm also correlate with anomeric protons suggesting that these signals correspond to the internal anomeric carbons.

To determine the molecular weight of a LMW-heparin a single reducing end anomeric carbon in $\sim 20$ internal anomeric carbons must be quantitated by $^{13}C$ NMR. The microheterogeneity and polydispersity of the LMW-heparin results in signal splitting and reduced sensitivity. This low sensitivity together with the low natural abundance of $^{13}C$ nuclei, and the low field strength magnets previously available, have made the determination of molecular weight by this technique problematic[19].

When the $^{13}C$ NMR spectrum of RD heparin-f was recorded in a proton noise decoupled mode or using an INEPT pulse sequence, the reducing-end anomeric carbon signals were insufficiently resolved from spectral noise (Fig. 2B). Using a greater scan rate or lower temperature did not appreciably increase the signal–noise ratio. When the experiment was performed using a DEPT pulse sequence, a significantly higher sensitivity (signal–noise ratio) was observed (Fig. 2C). Optimization of the DEPT pulse sequence using high gain resulted in further improvement in the sensitivity (Fig. 2D). The increased signal–noise ratio in the DEPT sequence probably arises due to low power selective decoupling of anomeric carbons, thereby reducing the spectral noise.

The DEPT spectra of LMW-heparins are as shown in Fig. 3 (anomeric region) and Fig. 4. These spectra demonstrate major differences in structural features of commercial LMW-heparins. Peaks due to the major residues of LMW-heparin ($\mathbf{Is}_{INT}$ and $\mathbf{A2S6s}_{INT}$, Fig. 1) can be easily identified[23,25]. Unsulfated iduronic acid C-1 gives signals at 102–103 ppm ($\mathbf{I}_{INT}$, Fig. 1) and glucuronic acid gives signals in the region 103–105 ppm ($\mathbf{G}_{INT}$, Fig. 1). The composition of the polysaccharide

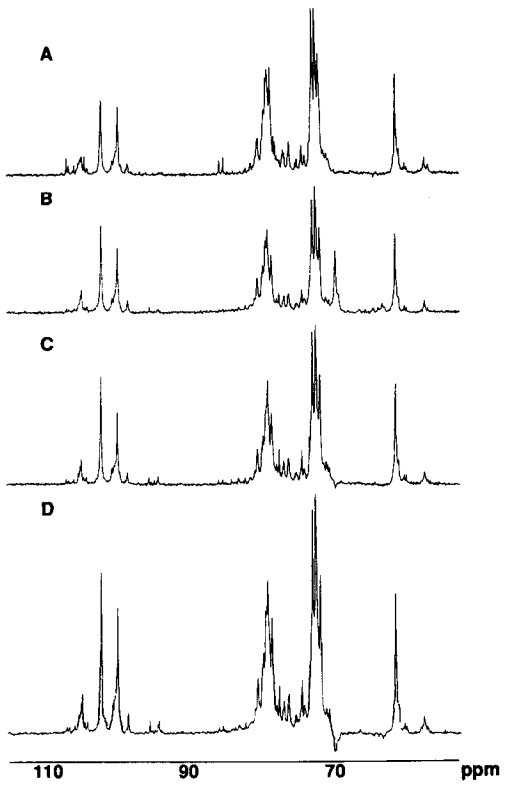


Fig. 2. $^{13}C$ NMR spectra of heparin and LMW-heparins recorded using various acquisition parameters. (A) Porcine mucosal heparin using DEPT pulse sequence. The spectrum was acquired with 64000 scans with 0.66 s recovery delay. Note no signals corresponding to the reducing end anomeric carbons are observed in the region 92–96 ppm. (B) RD heparin-f (LMW-heparin) using broad band decoupling method. The spectrum was acquired with 62000 scans and processed with 1.5 Hz line broadening factor. The decoupler was set to 2 W high decoupler power. (C) RD heparin-f using DEPT pulse sequence. The spectrum was acquired with 64000 scans and processed with 1.5 Hz line broadening factor. The proton decoupler channel was set to a decoupler power of 0.4 watts. Note the increased signal–noise ratio of reducing end groups in DEPT spectrum as compared to BB spectrum. Also note the higher resolution in DEPT spectrum. (D) RD heparin-g DEPT spectrum (96000 scans; 1.5 Hz LB) with higher gain.

chain of LMW-heparin in terms of different saccharide residues could be deduced by the signal intensity of each of these. In addition, the anomeric region also reflects the mode of preparation of these LMW-heparins. Logiparin (Fig. 3C) exclusively exhibits only one type of reducing end group, **A2s6s$_{RE}$** (Fig. 1) at 94 ppm. Logiparin is prepared through heparin lyase catalyzed partial $\beta$-eliminative depolymerization of porcine heparin[35]. The substrate specificity of heparin lyase I[36] results in homogeneity of both the reducing end **A2s6s$_{RE}$** and the nonreducing end groups, **ΔUA2S** (Fig. 1). Enoxaparin (Fig. 3A) is obtained through benzylation of heparin followed by $\beta$-elimination and alkaline hydrolysis[37]. This results in the formation of both uronic acid and glucosamine (**A2s$_{RE}$**, and **A2s6s$_{RE}$**, Fig. 1)

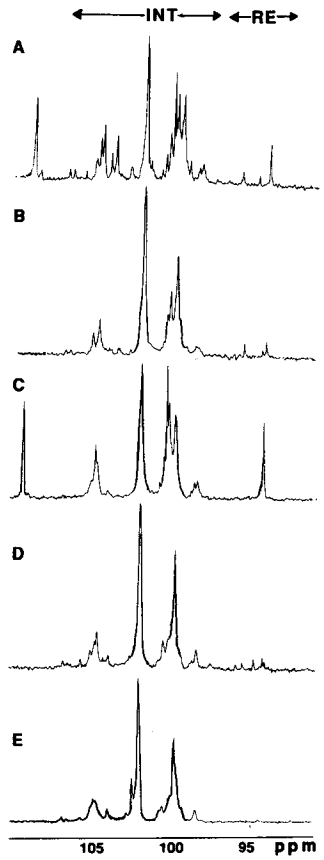


Fig. 3. DEPT spectra showing the anomeric carbon signal region for LMW-heparins. (A) Enoxaparin: assignments for the nonreducing end (C-4), reducing end (C-1), and internal (C-1) signals were made based on literature values[20,21,23,28]. The signal at 108 ppm corresponds to C-4 of unsaturated uronic acid residues at the nonreducing end of the polysaccharide chain. Three reducing end C- 1 signals are observed. The intense signal at 93 ppm corresponds to the C-1 of $\mathbf{A2s6s_{RE}}$, the signal at 94.5 ppm corresponds to $\mathbf{A2s_{RE}}$, and the signal at 95.5 ppm to C-1 of either $\alpha$-L-idopyranosyluronic or $\beta$-D-glucopyranosyluronic acid residue. Multiple C-1 resonances corresponding to internal sugars are observed between 98 and 107 ppm. The cluster of signals from 99–101 ppm correspond to the C-1 of $\mathbf{A2s6s_{INT}}$, $\mathbf{A2s_{INt}}$, $\mathbf{A2Ac6s_{INT}}$, and $\mathbf{A2Ac_{INT}}$ (assignments are interchangeable). The intense signal at 102 ppm is for the C-1 of $\mathbf{Is_{INT}}$. The cluster of signals from 103.5–105 ppm correspond to the C-1 of $\mathbf{I_{INT}}$ and those between 105.5 and 107 ppm to the C-1 of $\mathbf{G_{INT}}$. (B) Fluxum; (C) Logiparin; the sharp signal at 99.9 ppm corresponds to anomeric carbon of the nonreducing end unsaturated uronic acid residue. (D) RD heparin-c and (E) Fragmin. Note the absence of signals in the reducing end anomeric carbon region.

reducing end residues as shown by the anomeric carbon signals at 96.1, and 92.5 and 93 ppm, respectively. The oxidative degradation methods[38] for preparing RD heparin (Fig. 3D) and Fluxum (Fig. 3B) from porcine and bovine mucosal heparins, respectively, is nonspecific resulting in a multiplicity of reducing end residues, as suggested by anomeric carbons signals at 93.8, 94.0, 94.8, 95.5, and 96.1 ppm,

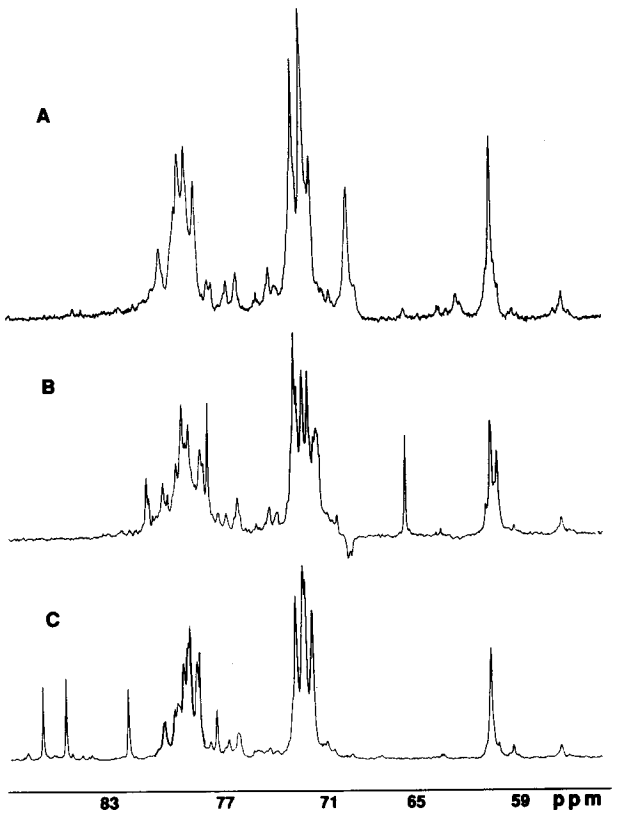


Fig. 4. The $^{13}C$ NMR spectra showing the C-2 to C-6 region of LMW-heparins. (A) RD heparin-g using the BB method; (B) DEPT spectrum for Logiparin; the signal observed at 65.6 ppm is assigned to the C-2 of nonreducing end unsaturated uronic acid residue; (C) DEPT spectrum for Fragmin; signals at 81.9, 85.5, and 86.9 ppm correspond to the C-5, C-4, and C-2 of the anhydromannitol ($\mathbf{Am}_{\mathbf{RE}}$, Fig. 1) reducing end residue.

similar to what is found in the parent heparin. The proportion of different types of reducing end groups in LMW-heparins can also be determined. Whereas, Enoxaparin exhibits a ratio of glucosamine to uronic acid type reducing end groups of 1:0.3, Fluxum exhibits a ratio of 1:0.8. The significantly different proportion of uronic acid reducing end groups in LMW-heparins reflect differences in the preparation of these drugs. Absence of signals in the 92–96 ppm region for Fragmin (Fig. 3E) suggests a different form of reducing end groups. Fragmin is produced by nitrous acid depolymerization of porcine mucosal heparin followed by reduction, which leads to an anhydromannitol ($\mathbf{Am}_{\mathbf{RE}}$ or $\mathbf{Am6s}_{\mathbf{RE}}$, Fig. 1) reducing end. The presence of this residue can be confirmed by three signals at 81.9, 85.6, and 87.1 ppm (Fig. 4C) belonging to C-5, C-2, and C-4, respectively, of the anhydromannitol residue[39]. These signals can also be used to determine the average molecular weight.

TABLE II

Disaccharide compositional analysis of LMW-heparins by capillary electrophoresis

| LMW-Heparin | Disaccharide composition (% of total) [a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | **3** [b] [401] [c] | **4** [503] | **5+6** [482] | **7** [601] | **8** [563] | **9** [563] | **10** [665] |
| RD heparin-a | 0.19 | 0.28 | 0.26 | 0.77 | 9.20 | 3.92 | 79.86 |
| RD heparin-b | 0.15 | 0.21 | 0.17 | 0.94 | 8.15 | 3.91 | 81.24 |
| RD heparin-c | 0.33 | 0.47 | 0.24 | 0.72 | 10.11 | 3.23 | 79.19 |
| RD heparin-d | 0.11 | 1.14 | nd [d] | 1.08 | 7.96 | nd | 86.54 |
| RD heparin-e | 0.62 | nd | nd | 1.00 | 4.42 | 3.88 | 83.84 |
| RD heparin-f | 0.15 | 1.21 | 0.28 | 0.14 | 11.92 | 1.61 | 78.17 |
| RD heparin-g | 0.18 | 0.72 | 0.22 | 1.21 | 10.46 | 3.76 | 78.63 |
| Fraxiparin | nd | 0.5 | 0.52 | 2.23 | 5.04 | 10.39 | 81.32 |
| Fluxum | nd | 0.54 | nd | nd | 0.80 | 22.50 | 76.16 |
| Fragmin | nd | 0.44 | 0.30 | 2.27 | nd | 4.47 | 92.51 |
| Logiparin | 0.03 | 0.22 | 0.26 | 0.24 | 1.48 | 3.88 | 85.31 |
| Enoxaparin | nd | 0.30 | 0.22 | 1.49 | 1.78 | 5.39 | 81.45 |

[a] Capillary electrophoretic analysis of LMW-heparins using 10 mM sodium borate and 50 mM boric acid buffer at pH 8.81 under 20130 V with gravity injection method. [b] Numbers correspond to formulas in Fig. 1. [c] Numbers in brackets are disaccharide molecular weights. [d] Not detected or not determined.

*Disaccharide compositional analysis of LMW-heparins.*—To accurately calculate the molecular weight of LMW-heparin using $^{13}C$ NMR it is also necessary to perform disaccharide compositional analysis. LMW-heparins were depolymerized using a mixture of heparin lyase I, heparin lyase II, and heparin lyase III under conditions which degrade the chains primarily to disaccharide products. The disaccharides were then quantitated by capillary electrophoresis[30]. The disaccharide compositional analysis is as shown in Table II.

Although the disaccharide compositional analysis of LMW-heparins is desirable for the accuracy of average molecular weights, in cases where this analysis is not available, a value of 625 mass units, representing the average molecular mass of a disaccharide unit, can be used.

*Molecular weight calculation for LMW-heparins.*—The number average molecular weight of LMW-heparin was deduced by eq *10*.

$$M_n = [((\text{signal ratio}/1.15) + 1)/2] \times \left(\sum P_i \times M_i/100\right) \qquad (10)$$

The factor 1.15 is correction factor for the differential relaxation rates; $P_i$ is the percentage of disaccharide i of molecular weight $M_i$. The signal ratio is the intensity of the total internal anomeric signals divided by the intensity of the total reducing end anomeric signals. The first term in parenthesis [((signal ratio/1.15) + 1)/2] transforms the proportion of internal residues to reducing end residues into the number of disaccharides constituting an average polysaccharide chain. The second term ($\sum P_i \times M_i/100$) gives the average molecular weight of a disaccharide residue. Equation *10* is of the same form as eq *2*, thus, yielding a number average molecular weight.

TABLE III

Average molecular weight of LMW-heparins by $^{13}C$ NMR (DEPT) spectroscopy

| LMW-heparin | Number of scans | LB [a] | Intensity ratio [b] | Ratio corr. [c] | Disacc. mass [d] | Molecular weight |
|---|---|---|---|---|---|---|
| RD heparin-a | 64256 | 1.5 | 24.90 | 21.65 | 613.02 | 6942 |
| RD heparin-b | 22560 | 3.0 | 22.20 | 19.30 | 616.31 | 6256 |
| RD heparin-c | 25088 | 3.0 | 23.50 | 20.43 | 610.92 | 6546 |
| RD heparin-d | 78720 | 1.0 | 26.50 | 23.04 | 633.02 | 7609 |
| RD heparin-e | 26176 | 3.0 | 27.50 | 23.91 | 612.80 | 7632 |
| RD heparin-f | 33024 | 3.0 | 20.27 | 17.63 | 615.67 | 5735 |
| RD heparin-g | 64176 | 1.5 | 22.70 | 19.74 | 604.89 | 6273 |
| Fraxiparin | 64182 | 3.0 | 20.90 | 18.18 | 646.16 | 6197 |
| Enoxaparin | 35232 | 3.0 | 21.31 | 15.34 [f] | 593.59 | 4850 |
| Fluxum | 27360 | 3.0 | 24.31 | 21.14 | 640.36 | 7089 |
| Fragmin | 92544 | 1.0 | 11.00 | 11.00 [e] | 657.75 | 3947 |
| Logiparin | 26016 | 1.0 | 15.50 | 11.16 [f] | 601.42 | 3657 |

[a] Line broadening factor used for Fourier transformation. [b] Signal intensity ratio ($S_{int}/S_{red}$) obtained from the DEPT spectra without any relaxation rates correction. [c] Corrected signal intensity ratio obtained after taking into account the relaxation rates (eqs 7 or 8). [d] Calculated disaccharide mass using the term $\sum P_i \times M_i/100$ (eqs *10* and *11*, see text and Table II). [e] Signal intensity ratio not corrected due to unquantified relaxation effects. [f] Signal intensity ratio taking into account the unsaturated uronic acid residue (eq *8*).

The $M_n$ values determined from eq *10* using DEPT (Table III) suggest that the commercial preparations of LMW-heparins differ considerably in molecular weight. The number average molecular weights of LMW-heparins range from 3000 (Logiparin) to 7000 (RD heparin). A major advantage of the DEPT technique is that it does not require oligosaccharide standards or a calibration curve. In addition, the $^{13}C$ NMR spectrum obtained using DEPT is valuable in identifying

TABLE IV

Number ($M_n$) and weight ($M_w$) average molecular weights of LMW-heparins by GPC–HPLC

| LMW-heparin | $M_n$ | $M_w$ | Polydispersity | Chains $< 2500$ $M_r$ (% of total) | Chains $> 10000$ $M_r$ (% of total) |
|---|---|---|---|---|---|
| RD heparin-a | 5901 | 8001 | 1.36 | 15.5 | 12.3 |
| RD heparin-b | 5607 | 7609 | 1.36 | 17.5 | 10.5 |
| RD heparin-c | 5503 | 7183 | 1.31 | 15.6 | 9.5 |
| RD heparin-d | 6305 | 8992 | 1.43 | 18.3 | 11.2 |
| RD heparin-e | 5966 | 8119 | 1.36 | 13.5 | 12.3 |
| RD heparin-f | 5264 | 7534 | 1.43 | 22.5 | 10.8 |
| RD heparin-g | 5156 | 6617 | 1.28 | 18.9 | 6.8 |
| Fraxiparin | 5324 | 6442 | 1.21 | 4.3 | 6.3 |
| Enoxaparin | 3217 | 4395 | 1.37 | 44.2 | 1.0 |
| Fluxum | 5414 | 7561 | 1.40 | 20.3 | 9.7 |
| Fragmin | 5379 | 6466 | 1.20 | 7.7 | 4.5 |
| Logiparin | 4273 | 5683 | 1.33 | 26.7 | 3.7 |

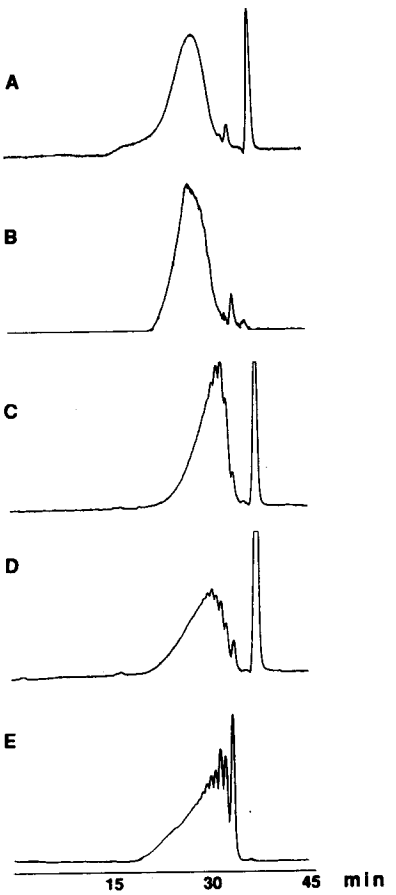


Fig. 5. GPC-HPLC chromatograms of LMW-heparins using TSK2000W and TSK3000W columns in series. The ordinate is absorbance at 206 nm with a maximum value of 0.05 AU. (A) Fraxiparin; (B) Fragmin; (C) Enoxaparin; (D) Logiparin; and (E) mixture of oligosaceharide standards prepared by partial, controlled enzymatic depolymerization of bovine lung heparin.

TABLE V

Number ($M_n$) and weight ($M_w$) average molecular weights of LMW-heparins by gradient PAGE

| LMW-heparin | $M_n$ | $M_w$ | Polydispersity | chains $> 2500\ M_r$ (% of total) | chains $> 10000\ M_r$ (% of total) |
|---|---|---|---|---|---|
| RD heparin-a | 5877 | 7713 | 1.31 | 11.5 | 7.9 |
| RD heparin-b | 5554 | 7403 | 1.33 | 14.4 | 6.4 |
| RD heparin-c | 5818 | 7414 | 1.28 | 9.9 | 6.7 |
| RD heparin-d | 5550 | 7530 | 1.36 | 17.5 | 7.2 |
| RD heparin-e | 6093 | 8606 | 1.41 | 14.6 | 10.4 |
| RD heparin-f | 5541 | 7089 | 1.28 | 4.3 | 5.4 |
| RD heparin-g | 5422 | 6817 | 1.26 | 12.1 | 4.0 |
| Fraxiparin | 5142 | 6656 | 1.29 | 13.1 | 7.5 |
| Enoxaparin | 4295 | 6059 | 1.41 | 28.7 | 5.2 |
| Fluxum | 5712 | 8165 | 1.48 | 15.9 | 13.5 |
| Fragmin | 6285 | 7975 | 1.27 | 1.2 | 13.3 |
| Logiparin | 4967 | 6863 | 1.37 | 20.4 | 8.7 |

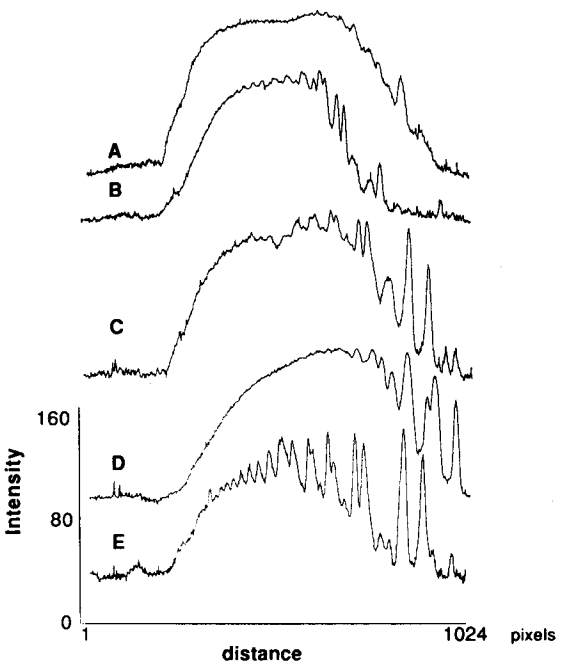


Fig. 6. Gel scan of gradient PAGE of LMW-heparins. The ordinate represents the intensity of the band which was set to a maximum value of 255 units, while the abscissa represents the distance of bands from the loading point as measured by the number of pixels. The maximum number of pixels (1036) corresponded to the total length of the gel. This number could be varied by about half the scale (512) without significantly affecting the results. (A) Fluxum; (B) Fragmin; (C) Logiparin; (D) Enoxaparin; and (E) mixture of oligosaccharide standards prepared by partial, controlled enzymatic depolymerization of bovine lung heparin.

minor differences in molecular structures in the LMW-heparins that could be valuable in explaining differences in the pharmacological behavior of these drugs[10]. The 16 h acquisition time required for the DEPT analysis is reasonably short making this technique particularly attractive. With the current availability of high field strength magnets (greater than 400 MHz) and more sophisticated software, together with greater sensitivity of new X-nuclei probes, the acquisition times could possibly be shortened to within 10 h without jeopardizing sensitivity.

The accuracy of the molecular weights of LMW-heparins measured using DEPT was confirmed using GPC–HPLC and gradient PAGE analysis. Despite the limitations of these techniques, the number average molecular weights determined using GPC-HPLC (Fig. 5, Table IV) and the gradient PAGE (Fig. 6, Table V) compare favorably to those obtained using $^{13}C$ NMR.

In conclusion, $^{13}C$ NMR in conjunction with disaccharide compositional analysis provides an excellent measure of the $M_n$ of LMW-heparins without requiring the use of molecular weight standards or calibration curves.

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