Molecular Weight of Heparin Using ^{13}C Nuclear Magnetic Resonance Spectroscopy

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Abstract □ Heparin is a polydisperse, heterogeneous polysaccharide that has been used as an anticoagulant for the past 50 years. The molecular weight determination of this important drug has traditionally relied on gel permeation chromatography, which requires the use of well-defined molecular weight standards that are not easily obtained. We have investigated the use of ^{13}C-NMR spectroscopy for measuring the number average molecular weight of heparin. The signal intensities of the reducing end and internal anomeric carbons, having distinctive chemical shifts in the ^{13}C-NMR spectrum, were used to determine the molecular weight. Distortionless enhancement polarization transfer was found to provide a better quantitation of signal intensities of anomeric carbons than broad band decoupling or selective decoupling of anomeric protons. Signal averaging over 300 000 transients, requiring approximately 48 h on a 360 MHz NMR spectrometer, resulted in the measurement of the number average molecular weight (~10 000 Da) of heparin. ^{13}C-NMR spectroscopy does not require the use of difficult to obtain molecular weight standards and thus is particularly well-suited for workers in the pharmaceutical industry.
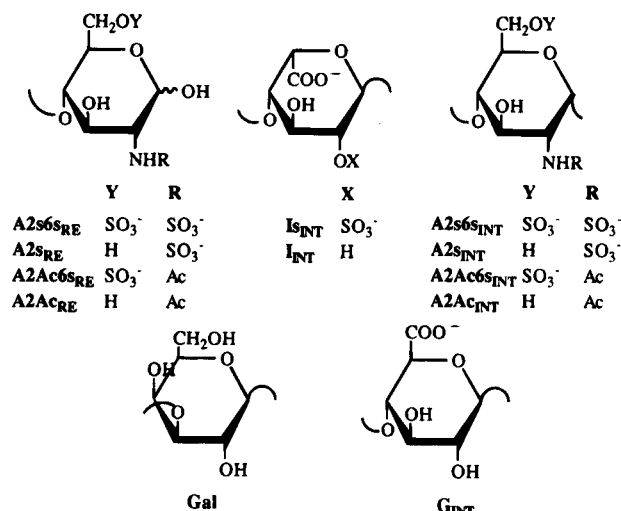

Figure 1—Saccharide constituents of the polymeric heparin. The subscripts RE and INT represent reducing end and internal residues. Gal represents galactose residues that occur in the portion of heparin near the peptide chain of proteoglycan heparin (found in raw heparin). The acidic groups (carboxylic and sulfates) have a sodium cation associated with them.

Heparin is a highly sulfated, polydisperse, heterogeneous polysaccharide composed of alternating 1→4 linked 2-deoxy-2-amidoglucopyranose and pyranosyluronic acid residues (Figure 1).[1-3] Raw heparin prepared by extraction from proteolyzed animal tissue and is then bleached to remove remnants of its protein core, affording pharmaceutical heparin.[1] Further processing, such as oxidative depolymerization, leads to a new drug product called low molecular weight heparin.[4,5] Both heparin and low molecular weight heparin are widely used as anticoagulant and antithrombotic agents, yet their precise chemical structure remains undetermined.[1,2]

A number of techniques have been explored for the determination of molecular weight of heparin. Viscometry,[6,7] ultracentrifugation,[8] and light scattering[9-11] are error prone due to the relatively small size (average molecular weight ~ 10 000) of the heparin chains. Gel permeation chromatography has been most often used for the pharmaceutical analysis of heparin and low molecular weight heparins.[12] However, this technique generally relies on ultraviolet detection at 206 nm, which is not selective for heparin, and consequently non-heparin excipients or contaminants may interfere with molecular weight determination. In addition, the low extinction coefficient of the carboxylate chromophore of heparin at 206 nm requires use of high sample concentrations, which may result in interchain interactions leading to inaccurate molecular weight determination. Polyacrylamide gel electrophoresis has also recently been used to estimate the molecular weight of heparin and low molecular weight heparins.[13] Both gel permeation chromatography and polyacrylamide gel electrophoresis, however, require the use of well-defined molecular weight standards.

Recently our laboratory reported the application of ^{13}C-NMR spectroscopy for the standard-free molecular weight determi-

nation of low molecular weight heparins (average molecular weight ~5 000).[14] The present report describes the first use of ^{13}C-NMR to determine the molecular weight of pharmaceutical heparin having an average molecular weight of ~10 000.

Experimental Section

Materials—Low molecular weight heparin was from Wyeth-Ayerst Research Laboratories, Rouses Point, NY. Porcine mucosal heparin was from Sigma Chemical Co. (St. Louis, MO). Raw heparin (Stage 12) was from Celsus (Franklin, OH). ^{2}H$_2$O (99.96% atom ^{2}H) and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) were from Aldrich Chemical Co. (Milwaukee, WI).

Methods—Heparin or low molecular weight heparin (~3 g) in 10 mL of deionized, distilled water at pH 7.0 was filtered through a 0.45 μm membrane and freeze-dried. The resulting solid was exchanged with ^{2}H$_2$O, prepared in 3 ml of ^{2}H$_2$O (99.996% atom %), and degassed overnight under a vacuum. The DEPT spectra were recorded using standard Bruker software in DISN85 version using an ASPECT 2000 computer on a WM360 Bruker NMR spectrometer (Switzerland). The following pulse sequence was used: ^{1}H, 90°–Δ–180°–Δ–90°–Δ–decouple; ^{13}C, 90°–Δ–180°–Δ–FID. The delay (Δ) was set to 1/2J and a $^1J_{CH}$ of 170 Hz was used for optimizing the polarization transfer for tertiary carbons. A relaxation delay of 0.656 s and an acquisition time of 0.344 s were used. This corresponded to a pulse repetition time of 1 s. The longitudinal relaxation times of anomeric protons and anomeric carbons[14] are less than 1 s and 0.5 s, respectively. This permits good polarization transfer and, hence, signal intensity enhancement. The decoupler frequency was set at the center of anomeric proton multiplet (6826 Hz) with 0.4 W of decoupler power. The spectra were recorded using 16K data points and a spectral width of 23809 Hz. The free induction decays (FIDS) were processed off-line using a 1.0–3.0 Hz line broadening factor. The ^{13}C-NMR spectra were referenced by setting the carbon of TSP to 0.0 ppm.
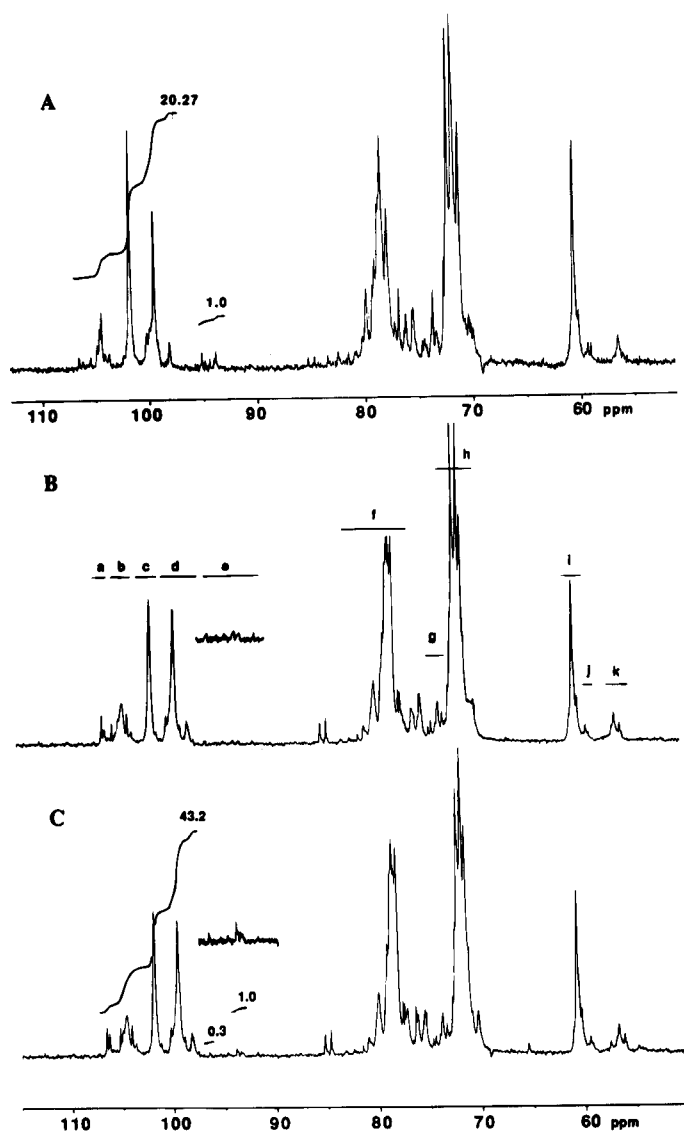
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Figure 2—^{13}C NMR spectra of heparins. (A) The DEPT spectrum of a low molecular weight heparin. (B) The DEPT spectrum of porcine mucosal heparin. (C) The DEPT spectrum of raw heparin. Insets in B and C show the region of interest corresponding to the anomeric carbons of reducing end residues. All spectra were obtained at room temperature; see ref 14 for additional experimental details. Labels on the groups of resonances correspond to the carbons of residues (see Figure 1) as follows: a, C-1 of GlcA$_{INT}$; b, C-1 of IdoA$_{INT}$; c, C-1 of IdoAp2s$_{INT}$; d, C-1 of GlcNp2s6s$_{INT}$ and GlcNp2Ac6s$_{INT}$; e, C-1 of reducing end glucosamine and uronic acid residues; f, C-2 and C-4 of A2s$_{INT}$, A2s6s$_{INT}$, I$_{INT}$, and Is$_{INT}$; g, C-3 and C-5 of I$_{INT}$ and G$_{INT}$; h, C-3 and C-5 of A2s$_{INT}$, A2s6s$_{INT}$, and Is$_{INT}$; i, C-2 of A2s6s$_{INT}$; j, C-2 of A2s3s6s$_{INT}$; k, C-2 of A2Ac$_{INT}$ and A2Ac6s$_{INT}$.

Results and Discussion

The ^{13}C NMR spectra of heparin and heparin-derived oligosaccharides have been well studied.[15-19] Many resonances in heparin have been assigned, particularly signals arising from the anomeric carbons of the internal and reducing end residues (Figure 1). The reducing end anomeric carbons resonate in the region 91–96 ppm while the anomeric carbons of the internal saccharide residues resonate between 97 and 105 ppm[14] (Figure 2). We hypothesized that the average degree of polymerization (dp) could be arrived at if the ratio of reducing end residues to internal residues in heparin could be determined. The number average molecular weight could then be estimated from the dp without the use of molecular weight standards.

Quantitative ^{13}C-NMR spectroscopy requires a detailed knowledge of the longitudinal relaxation times and the nuclear Overhauser enhancements (NOE) for the resonances being studied.[20] In ^{13}C-NMR, the probability that an adjacent carbon is a ^{13}C is <1% and hence relaxation by another carbon is negligible. Thus, the NOE for observing ^{13}C signals is principally due to heteronuclear dipolar relaxation and is a function of the gyromagnetic ratios of the carbons and protons. Since an equal number of protons are involved in relaxing the internal and reducing end anomeric carbons, it is reasonable to assume an equal NOE for the two due to directly bonded protons. Differences in the NOE for internal and reducing end residues due to differences in rotational correlation rates are extremely difficult to measure due to the heterogeneity of polydisperse heparin. These differences, however, are expected to be insignificant. Heparin is a relatively small linear polymer, and unlike globular polymers of extremely high molecular weight, heparin should have a nearly isotropic tumbling rate. Thus, utilizing a proton decoupling channel of sufficient power, applied for sufficient duration, the NOE for internal and reducing end anomeric carbons in heparin should be equal. Hence, in quantitating the anomeric carbons, the NOE was assumed to be the same for both the internal and reducing end anomeric carbons. Thus a simplified equation for calculating carbon intensities was used.

The signal intensity (S) of a nuclei (i) at any time t is related to its relaxation rate (T_{1i}) by eq 1:

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

$S_i(0)$ represents the signal intensity of nuclei i at time 0. The relaxation rates determined using a heparin-derived oligosaccharide[14] suggested that the average longitudinal relaxation time for internal anomeric carbons (241 ms) was slightly longer than that for reducing end anomeric carbons (220 ms). The ratio of the signal intensities for the reducing end ($S_{red}(\tau)$) and the internal ($S_{int}(\tau)$) anomeric carbons at any time (τ) could, hence, be derived (eq 2):[20]

$$S_{red}(\tau)/S_{int}(\tau) = [S_{red}(0)/S_{int}(0)]_e{}^{-\tau\{1/T_{1red} - 1/T_{1int}\}} \qquad (2)$$

At time 0, the signal intensities for both the reducing end and internal anomeric carbons would be equal. By substituting an acquisition time of 0.344 for τ, eq 2 provides the signal intensity ratio for anomeric internal and reducing end carbons in heparin (eq 3):

$$S_{int}/S_{red} = 1.15 \qquad (3)$$

Equation 3 indicates that the intensity of reducing end anomeric carbons first need to be increased by a factor 1.15 before determining the ratio of signals intensities arising from the anomeric carbons of the internal and reducing end residues in heparin.

The ^{13}C-NMR spectrum of a low molecular weight heparin (oxidatively depolymerized heparin[2]) was first recorded using the broad band (BB) decoupling method (data not shown). However, no signals were observed in the region 91–96 ppm. The distortionless enhancement polarization transfer (DEPT) experiment is expected to be more sensitive than the BB decoupling method.[21] The DEPT experiment relies on the enhancement of the intensity of the resonance of low magnetogyric ratio, such as carbon-13, through spin–spin coupling.[21] Typically the polarizing nucleus is hydrogen and its spin–spin coupling leads to a 4-fold enhancement in signal intensity of the attached carbon. In addition, successive cycles in a time-averaged sequential pulse experiment may be repeated

with relaxation delays based on the longitudinal relaxation times (T_1) of protons, rather than on that of carbon-13 being observed. The different T_1 values of anomeric protons of a heparin-derived tetrasaccharide[14] were less than 1 s, and hence, the pulse repetition was set to the same value in the DEPT experiment. Furthermore, the DEPT spectra were recorded with ^{1}H decoupling during the acquisition of FID, resulting in additional signal enhancement. Figure 2A shows the DEPT where a number of signals corresponding to the anomeric carbons of the reducing end residues were obtained. After normalizing the signal intensity using eq 3, an intensity ratio was obtained from which a dp of 18 can be calculated.

The intensity of the signals corresponding to the anomeric carbons of the reducing end residues for heparin is expected to be substantially lower than that of low molecular weight heparin. In addition, the long time for data collection, needed to observe the reducing end anomeric carbons, cause field drift, resulting in a loss of these minor signals. The concentration of the heparin sample cannot be increased due to substantial broadening of ^{13}C resonances associated with the increased viscosity. While a higher temperature of acquisition (37 or 50 °C) alleviates the problem of signal broadening, it also leads to increased baseline noise, resulting in complete or partial signal loss. The ^{13}C NMR spectra of two heparins were recorded with a DEPT pulse sequence at room temperature (Figure 2B,C). The inset in Figure 2B,C shows the expanded region of interest (91−96 ppm). Approximately 300 000 transients were necessary to observe the reducing end anomeric carbon signals of heparin. The intensity ratio of internal to reducing end anomeric carbons (again taking into account eq 3) corresponds to a dp of 30 for porcine mucosal heparin and 29 for raw heparin.

The average mass of constitutive monosaccharides is required to convert the degree of polymerization to the average molecular weight. Since heparin is an alternating copolymer of glucopyranose and pyranosyluronic acid residues (Figure 1), the average disaccharide mass is used to determine molecular weight. A heparin preparation is enzymatically depolymerized to a mixture of disaccharides, which is analyzed by capillary electrophoresis using eight standard disaccharides of known structure. The average disaccharide mass is then calculated from the mass and mol % of each disaccharide present in the depolymerized heparin sample.[14,22,23] For most heparins, the average disaccharide mass is found to be approximately 640 Da.

The average molecular weight of heparin is calculated from ^{13}C-NMR signal intensities using eq 4:

$$M_n = [\{S_{int}/(S_{red} \times 1.15) + 1\}/2] \times$$

[average disaccharide mass] (4)

The first term transforms the proportion of internal to reducing end residues into the number of disaccharides constituting an average polysaccharide chain. The equation resembles the equation for calculation of the number average molecular weight (M_n), and hence, the ^{13}C-NMR method leads to M_n.

The number averaged molecular weight of low molecular weight heparin, porcine mucosal heparin, and raw heparin were found to be 5430, 9670, and 9248, respectively, using ^{13}C-NMR technique (Table 1). These molecular weights are comparable to those obtained by polyacrylamide gel electrophoresis and gel permeation chromatography in our laboratory (Table 1)[13,14] as well as to values obtained by others on similar samples.[12]

The weak signal intensity of the reducing end carbons in heparin (even after collecting 300 000 transients) results in considerable error in determination of M_n when using an

Table 1—Average Molecular Weight of Heparins by ^{13}C NMR (DEPT) Spectroscopy

sample	number of transients	LB[a]	intensity ratio[b]	corrected ratio[c]	molecular weight[d]
low molecular weight heparin	33024	3.0	20.27	17.63	5430[e]
porcine mucosal heparin	211968	3.0	34.75	30.22	9670[f]
raw heparin	314188	1.5	33.23	28.90	9248[f]

[a] Line broadening factor. [b] Signal intensity ratio (S_{int}/S_{red}) obtained from the DEPT spectrum without correction. [c] Signal intensity ratio corrected for differences in relaxation rates. [d] The M_n of low molecular weight heparin was 5541 and 5264, as measured by gradient PAGE and gel permeation chromatography, respectively.[14] The M_n of porcine mucosal heparin and raw heparin as estimated to be 11 000 by gradient PAGE.[13] [e] The disaccharide mass for low molecular weight heparin was 616 by capillary electrophoresis.[14] [f] The number average molecular weight using an average disaccharide mass of 640 Da.

instrument operating at 360 MHz. The error in M_n is estimated as ±500 using this instrument. The availability of higher sensitivity probes as well as higher field strengths should considerably reduce the error in measuring M_n. Alternatively, more sensitive instrumentation can be used to reduce the acquisition times required for sample analysis.

The ^{13}C-NMR method of determining the average molecular weight of heparin enjoys the important advantage of not requiring the preparation of structurally defined oligosaccharide standards. Preparation of such oligosaccharides, particularly those having a degree of polymerization of >20, would require an enormous effort. Such standards have not been prepared to date. In addition, ^{13}C-NMR can identify structural changes occurring during heparin processing, may be helpful in differentiating lot to lot variation, and may also lead to an improved understanding of heparin's important biological activities.

Conclusions

This study demonstrates that ^{13}C-NMR can be used to determine the number average molecular weight of heparin. This method does not require the use of standards and can be performed on commercial heparin samples having a molecular weight of 10 000.

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Acknowledgments