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 anionic 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 anionic carbons than broad band decoupling or selective decoupling of anomic 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 existing molecular weight standards and thus is particularly well-suited for workers in the pharmaceutical industry.

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

A number of techniques have been explored for the determination of molecular weight of heparin. Viscometry, $^{1}$ ultracentrifugation, $^{8}$ and light scattering 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. 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. Polycrylamide gel electrophoresis has also recently been used to estimate the molecular weight of heparin and low molecular weight heparins. Both gel permeation chromatography and polycrylamide 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-

Figure 1 — Sugar 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.

![Figure 1](image-url)

Materials — Low molecular weight heparin was from Wyeth-Ayerst Research Laboratories, Rouseys, NY. Porcine mucosal heparin was from Sigma Chemical Co. (St. Louis, MO). Raw heparin (Stage 12) was from Cellexus (Franklin, OH). H2O (99.96% atom H) and 3-(trimethylsilyl)propionic-2,2,3,3,-d 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 $^{1}$H2O, prepared in 3 mL of H2O (99.96% atom %) and degassed overnight under a vacuum. The DEPT spectra were recorded using standard Bruker software in DISN65 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 1.27 and a V/F 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 anomic protons and anomic carbons 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 anomic 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.
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. In $^{12}$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 anomic 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 anomic carbons in heparin should be equal. Hence, in quantitatively the anomic carbons, the NOE was assumed to be the same for both the internal and reducing end anomic carbons. Thus a simplified equation for calculating carbon intensities was used.

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

$$S_i(t) = S_i(0)e^{-T_{1i}}$$

Equation 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 anomic carbons (241 ms) was slightly longer than that for reducing end anomic carbons (220 ms). The ratio of the signal intensities for the reducing end ($S_{\text{red}}(t)$) and the internal ($S_{\text{int}}(t)$) anomic carbons at any time ($t$) could, hence, be derived (eq 2):$^{20}$

$$S_{\text{red}}(t)/S_{\text{int}}(t) = [S_{\text{red}}(0)/S_{\text{int}}(0)]e^{-t/(T_{1\text{red}} - T_{1\text{int}})}$$

Equation 2

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

$$S_{\text{int}}/S_{\text{red}} = 1.15$$

Equation 3

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 anomic carbons of the internal and reducing end residues (Figure 1). The reducing end anomic carbons resonate in the region 91–96 ppm while the anomic 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.

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 A and B show the region of interest corresponding to the anomic 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: c, C-1 of GlcA; c, C-1 of IdoA; c, C-1 of IdoApn2; d, C-1 of GlcA2s6s; and GlcA2s6s2; e, C-1 of reducing end glucosamine and uronic acid residues; f, C-2 and C-4 of A2s, A2s6s, A2s6s2, and Isn2; g, C-3 and C-5 of Isn; h, C-3 and C-5 of A2s, A2s6s, and Isn; i, C-2 of A2s6s; j, C-2 of A2s6s2; k, C-2 of A2s6s2; and A2s6s2.
Table 1—Average Molecular Weight of Heparins by $^{13}$C NMR (DEPT) Spectroscopy

<table>
<thead>
<tr>
<th>sample</th>
<th>number of transients</th>
<th>LB$^a$</th>
<th>intensity ratio$^b$</th>
<th>corrected ratio$^c$</th>
<th>molecular weight$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>low molecular weight heparin</td>
<td>33024</td>
<td>3.0</td>
<td>20.27</td>
<td>17.63</td>
<td>5430$^a$</td>
</tr>
<tr>
<td>porcine mucosal heparin</td>
<td>211968</td>
<td>3.0</td>
<td>34.75</td>
<td>30.22</td>
<td>9617$^a$</td>
</tr>
<tr>
<td>raw heparin</td>
<td>314188</td>
<td>1.5</td>
<td>33.23</td>
<td>28.90</td>
<td>9248$^a$</td>
</tr>
</tbody>
</table>

$^a$ Line broadening factor. $^b$ Signal intensity ratio ($S_{\text{dd}}/S_{\text{red}}$) 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 5234 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.$^{13}$ The disaccharide mass for low molecular weight heparin was 616 by capillary electrophoresis.$^{14}$ (The number average molecular weight using an average disaccharide mass of 540 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 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.

References and Notes


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