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Measurement of the Antithrombin III Binding Sites in Low Molecular Weight Heparins by ^{13}C NMR and Capillary Electrophoresis

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Abstract

Low molecular weight heparins, prepared from the controlled chemical or enzymatic depolymerization of the heparin polysaccharide, are currently replacing heparin as the clinical anticoagulant/antithrombotic agent of choice. A principal mechanism of action of these agents is through the binding of antithrombin III, a plasma serine protease inhibitor, to specific pentasaccharide sequences in these polysaccharides. The content of antithrombin III-binding pentasaccharide sequences within low molecular weight heparins vary, making these drugs bio-inequivalent. Currently, the only way to examine the content of these pentasaccharide sequences within a low molecular weight heparin preparation relies on bioassay.

This study examined both ^{13}C NMR spectroscopy and oligosaccharide analysis by capillary electrophoresis as alternative measures of the content of antithrombin III binding sites within a variety of low molecular weight heparins.

The number of antithrombin III binding sites per chain, measured by ^{13}C NMR spectroscopy, correlated with the antithrombin III mediated anti-factor Xa activity of various low molecular weight heparins.

Low molecular weight (LMW) heparins are prepared by the controlled chemical or enzymatic depolymerization of heparin, a highly sulphated, polydisperse polysaccharide composed of alternating 1·4 linked glucosamine and hexuronic acid residues (Linhardt & Loganathan 1990; Linhardt 1992). LMW heparins have largely replaced heparin as antithrombotic agents in Europe several have been recently approved by the United States Food and Drug Administration. LMW heparins are claimed to have reduced side-effects (a higher safety/efficacy ratio), more predictable pharmacological action, sustained antithrombotic activity and better bioavailability than heparin (Fareed et al 1988). Two important characteristics of LMW heparin are believed to influence their improved properties, their low molecular weight (5000–8000 Da) and their enhanced ratio of antithrombin III (ATIII)-mediated anti-factor Xa (FXa) to anti-factor FIIa (FIIa) activity (Fareed et al 1988). The altered anti-FXa to anti-FIIa ratio is primarily because many of the LMW-heparin chains are of insufficient size to bind both FIIa and ATIII for full expression of anti-FIIa activity (Lindahl et al 1983). These LMW heparin chains, however, are of the required size to exhibit full anti-FXa activity since this activity only requires the presence of an intact ATIII pentasaccharide binding site (Choay et al 1983).

Other characteristics associated with LMW-heparin, including differences in their primary structure or saccharide sequence and their end-group residues have been studied in less detail. Methods for oligosaccharide mapping of LMW heparin (Linhardt et al 1990), comparable to peptide mapping of proteins, compositional analysis of heparins (Ampofo et al 1991) and end group analysis of LMW-heparins (Desai & Linhardt 1994) have been recently introduced by our laboratory. Pharmaceutical companies that manufacture LMW heparin currently assess each new batch of product using anti-FXa assay and gel permeation high-performance liquid chromatography (HPLC). There is a clear need to establish a method for use in the pharmaceutical industry, which does not rely on a bioassay, to estimate the number of ATIII binding sites within a particular LMW heparin. This communication examines the use of ^{13}C nuclear magnetic resonance spectroscopy (NMR) and capillary electrophoresis to estimate the number of ATIII binding sites within LMW heparins.

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Materials and Methods

Materials

Porcine intestinal heparin (sodium salt, 145 USP units mg^{-1}) was from Hepar (Franklin, OH). Raw heparin (unbleached, 158 USP units mg^{-1}) was from Celsus Laboratories (Cincinnati, OH). Low molecular weight heparins Fraxiparin (CY216) (Choay Laboratories, Paris, France), Fragmin (KABI2165) (KabiVitrum, Stockholm, Sweden), Logiparin (LHN-1) (Novo Industries, Franklin, OH), and Enoxaparin (PK10169) (Pharmuka Laboratories, Gennevilliers, France), were obtained from the manufacturers and used as such. RD heparins a–e (lot nos 13390, 325–1, 7200–20a, O39PR91, and O45PR91) were obtained from Wyeth-Ayerst Research Laboratories (Rouses Point, NY) and used as such. Homogeneous,

structurally defined heparin-oligosaccharide standards were prepared from porcine intestinal heparin using heparin lyase I (Rice & Linhardt 1989; Linhardt et al 1986, 1992). Heparin lyase I, having an activity of 130 units mg^{-1} against heparin was prepared in our laboratory and purified to homogeneity (Lohse & Linhardt 1992). FXa was from Enzyme Research Laboratories (South Bend, IN), ATIII was from KabiVitrum and Diagnostica Stago (Asnieres, France) and chromogenic substrates was from American Diagnostica (Greenwich, CT). ^2H_2O (99·9 and 99·96% atom ^2H) and TSP were from Aldrich Chemical Co., Milwaukee, WI. Sodium borate (decahydrate, 99%) was from Fisher Scientific (Fair Lawn, NJ), boric acid (electrophoresis grade) was from Mallinckrodt (Paris, KY) and sodium dodecyl sulphate (99%) was from BDH Chemicals (Poole, UK). All other chemicals were reagent grade.

Instrumentation

^{13}C NMR experimentation was performed on a WM 360 NMR spectrometer from Bruker Spectrospin (Switzerland). Capillary electrophoresis was performed using a Dionex Capillary Electrophoresis System (Model I) with Advanced Computer Interface, equipped with high voltage power supply capable of constant or gradient voltage control from Dionex Corporation (Sunnyvale, CA). The uncoated silica capillaries (75 or 50 mm i.d., 69 cm total length) were from Dionex.

Preparation of LMW heparins for ^{13}C NMR

LMW heparins (~3 g) were dissolved in 6 mL deionized water and the pH of the solution was adjusted to 7·0. The solution was filtered through 0·45-μm 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 and the final freeze-dried solution was dissolved in 3 mL ^2H_2O (99·96% atom ^2H). The final solution was degassed overnight under vacuum and stored at −20°C.

^{13}C NMR spectroscopy of LMW heparins

The DEPT spectra were recorded at 360 MHz using the standard Bruker software in DISN85 version on an Aspect 2000 computer. The following pulse sequence was used: ^1H, 90°-F-180°-F-90°-F-decouple; ^{13}C, 90°-F-180°-F-FID (Desai & Linhardt 1995). The delay (F) 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 are less than 1 s and 0·5 s, respectively (Desai & Linhardt 1994). This permits good polarization transfer and, hence, signal intensity enhancement. The decoupler frequency was set at the centre of the anomeric proton multiplet (6826 Hz) with 0·4 W of decoupler power. The spectra were recorded using 16 000 data points and a spectral width of 23 809 Hz. The free induction decays (FIDs) were processed off-line using a 1·0–3·0 Hz line broadening factor. Typically 20 000–90 000 FIDs were acquired and Fourier-transformed using an exponential multiplication factor of 1·0–3·0 Hz. The relaxation times of the C-2 resonances from 3-sulphated and 3-unsulphated residues of glucosamines were assumed to be equal for signal intensity calculations. The proton noise decoupled ^{13}C NMR spectra of LMW heparins were recorded with similar parameters and were referenced by setting TSP to 0 ppm.

Preparation of LMW heparin samples for enzymatic depolymerization

Stock solutions of LMW heparins for capillary electrophoresis analysis were prepared by dissolving the bulk drug at approximately 20 mg mL^{-1} concentration in deionized, distilled water and filtering through a 0·45-μm membrane. The pH of the solution was adjusted to 7·0 with 0·1 M NaOH. The filtrate was freeze-dried and reconstituted to an exact concentration of 20 mg mL^{-1} with distilled, deionized water.

Heparin lyase I depolymerization of LMW heparin

Heparin lyase I was added (25 m units mg^{-1} substrate) to a solution of LMW heparin sample in 5 mM sodium phosphate buffer (500 μL mg^{-1} substrate) at pH 7·0 containing 100 mM sodium chloride. 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 heparin lyase I 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 mg mL^{-1}.

Capillary electrophoresis analysis of LMW heparin

Separation and analysis of the completely depolymerized LMW heparins were carried out using a fused silica (externally coated except where the tube passed through the detector) capillary. A new capillary was activated by sequentially washing with 0·1 M phosphoric acid, 0·5 M sodium hydroxide, deionized, distilled water, and run buffer (10 mM sodium borate containing 50 mM sodium dodecylsulphate at pH 8·8) before use. The sample was injected using hydrostatic pressure (60-cm head height, 75-s injection period) to give ~25 nL total volume of injected solution. The electrophoresis was performed using 20 kV constant voltage. The detection system consisted of a variable-wavelength UV detector operating at 232 nm. The analysis of data was performed using the software package from Dionex. The disaccharide and oligosaccharide peaks were identified by injecting a mixture of well-characterized oligosaccharide standards. Peak due to hexasaccharide **2** was confirmed by co-injection with an authentic sample. The mole percent of ATIII binding sites was assumed to be equal to the mole percent of hexasaccharide **2** obtained from the total peak area of the electropherogram.

Anti-FXa assay

Anti-FXa activity was measured as previously described (Walenga et al 1985, 1986) by an automated chromogenic method.

Results

Stock solutions of LMW heparin were prepared from bulk drug at a concentration of 20 mg mL^{-1} based on weight.

The anti-FXa activity of each LMW heparin sample was measured and values are shown in Table 1. Each LMW heparin sample was exchanged with ^2H_2O and its ^{13}C NMR spectra recorded. A typical spectrum required 12–18 h to acquire at 360 MHz and is shown in Fig. 1. The peaks from 58–59 ppm correspond to the C-2 signals of the 2,3,6-trisulphated (and 2,3-disulphated) glucosamine residue found in the centre of the ATIII pentasaccharide binding site (Fig. 2, structure **1**). The signals at 55–56 ppm and 59–60·5 ppm correspond to the C-2 signals of the *N*-acetylated and *N*-sulphated glucosamine residues that are not 3-*O*-sulphated and are located outside of the ATIII binding site. By comparing the area corresponding to the C-2 of the 3-*O*-sulphated glucosamine residues with the area corresponding to the total area for all glucosamine C-2 resonances, the number of ATIII binding sites/chain of LMW heparin was calculated (Table 1). A plot of the number of ATIII binding sites/chain as a function of anti-FXa activity is linear, having a correlation of 0·82 (Fig. 3a).

Each LMW heparin was treated with heparin lyase I and analysed by capillary electrophoresis to obtain a quantitative oligosaccharide map. A typical capillary electropherogram is shown in Fig. 4. The peak having a migration time of 21·6 min corresponds to hexasaccharide **2** (prepared and structurally characterized in our laboratory (Linhardt et al 1986)) containing a portion of ATIII binding site 1 (Fig. 4, peak 2). The area of this peak was used to calculate the mole percent of ATIII binding sites in each LMW heparin (Table 1). The large, broad peak at 23·5 min is assigned to a trisulphated disaccharide arising from the major (75–90 mole %) repeating unit in heparin, →)α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S(1→ (where IdoAp is idopyranosyluronic acid, GlcNp is 2-deoxy-2-aminoglucopyranose and S is sulphate).

The percent of the total peak area corresponding to peak 2, was determined for each LMW heparin. When all the LMW heparins were analysed and the mole % of hexasaccharide **2** was plotted as a function of anti-FXa activity, no correlation was obtained (Fig. 3b). A modest correlation was observed, however, when LMW heparins prepared by a single manufacturer were examined using this method.

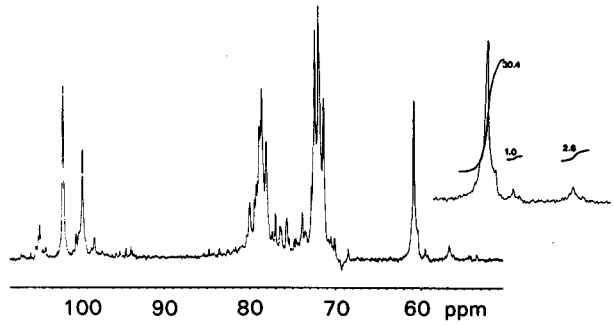

FIG. 1. DEPT spectrum of RD hep-a at 360 MHz. Inset: Peaks in the region 58–59 ppm corresponds to the C-2 of the 2,3,6-trisulphated glucosamine and 2,3-disulphated glucosamine residues. Peaks in the region 55–56 and 59–60·5 ppm correspond to C-2 of *N*-acetylated and *N*-sulphated glucosamine residues not 3-*O*-sulphated.

Discussion

Despite the great importance and widespread use of LMW heparins, the only convenient method to determine the potency of these agents is by bioassay. Both coagulation-based assays and chromogenic assays can be interfered with by the presence of drugs or endogenous substances that exhibit anticoagulant activity. In addition, the inherent variability of bioassays also limits their utility in evaluating batches of LMW heparins prepared by pharmaceutical companies. Anti-FXa activity is thought to be directly related to the molar concentration ATIII pentasaccharide binding sites within a particular LMW heparin (Casu et al 1981). Thus a chemical, enzymatic or spectroscopic assay that could rapidly and accurately quantify the number of ATIII pentasaccharide binding sites within a LMW heparin would be extremely valuable.

The ATIII pentasaccharide binding site found in porcine mucosal heparin and LMW heparins derived from the same tissue corresponds to structure **1** (Fig. 2). Structural variation can occur in this binding site (Loganathan et al 1990) without significant change in binding affinity or biological activity. For example, the centre glucosamine residue in **1** can be lacking a 6-*O*-sulphate group. Alternatively, the non-reducing terminal glucosamine can

Table 1. Concentration of ATIII binding sites in LMW heparins.

Sample	M_w Wt[a]	Weighted disac. mass[a]	(3S)-C-2/C-2 Ratio[b]	No. of ATIII sites[c]	% **2** by capillary electrophoresis[d]	Anti-FXa activity (units mg^{-1})
RD hep-a	7414	611	0·030	0·40	5·76	88
RD hep-b	7530	633	0·027	0·37	6·28	88
RD hep-c	8606	613	0·037	0·60	4·41	168
RD hep-d	7089	616	0·032	0·41	7·12	140
RD hep-e	6817	605	0·023	0·29	6·46	84
Fraxiparin	6656	646	0·027	0·35	8·48	104
Enoxaparin	6059	594	0·031	0·36	7·25	136
Fragmin	7975	658	0·072	0·76	3·06	248
Logiparin	6863	601	0·035	0·47	8·09	104
Raw heparin	nd[e]	nd	0·027	nd	7·44	88

[a]Weight average M_r and weighted disaccharide mass were obtained using gradient PAGE (Edens et al 1992) and capillary electrophoresis (Ampofo et al 1991; Desai & Linhardt 1994). [b]Intensity ratio of C-2 of 2,3,6-trisulphated and 2,3-disulphated glucosamine to total C-2 signals of all *N*-acetylated and *N*-sulphated glucosamines. [c]Number of 2,3,6 trisulphated and 2,3-disulphated glucosamine residues per chain using measured M_w values, [d]Percent moles of **2**, [e]Not determined.

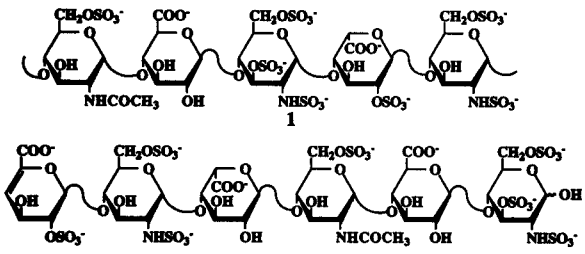

FIG. 2. The structure of the major ATIII pentasaccharide binding site **1** and a hexasaccharide **2** containing a portion of this binding site afforded on treating heparin or LMW heparin with heparin lyase I.

be *N*-sulphated instead of *N*-acetylated and both adjacent iduronic acid residues can be 2-*O*-sulphated.

Neville et al (1989) showed that ^{1}H NMR could be used to examine heparins for contaminating dermatan sulphate by quantifying the amount and types of the intensity of the *N*-acetylated hexosamine residues within a heparin preparation. Tachibana et al (1990) extended the application of ^{1}H NMR, demonstrating a correlation of *N*-acetyl signal in porcine mucosal heparin to anticoagulant activity as measured according to the XIth Japanese Pharmacopoeia. Although a correlation was observed ($r=0{\cdot}86$), it has been well established that some *N*-acetylated glucosamine residues are found outside the ATIII pentasaccharide binding site (Merchant et al 1985; Linhardt et al 1992).

The current study utilizes the high resolution of ^{13}C NMR to select a signal corresponding to the C-2 of the 2,3,6-trisulphated glucosamine residue (or the 2,3-disulphated glucosamine residue) found exclusively in the ATIII pentasaccharide binding site (Choay et al 1980). The area of this signal was compared with the total area of the C-2 signals of 2,6-disulphated glucosamine residues present to calculate the number of ATIII binding sites present in each LMW heparin (Table 1). These data were plotted against the anti-FXa activity of each LMW heparin (Fig. 3a). The technique of broad band decoupling utilizes high power to eliminate proton noise leading to lowered signal-to-noise ratio in the resulting ^{13}C spectrum in comparison with distortionless enhancement polarization transfer (DEPT) which uses a low-power selective decoupling. The DEPT spectra gave a better correlation.

The correlation of anti-FXa activity to the number of ATIII binding sites by the ^{13}C NMR method ($r=0{\cdot}82$) was good. The relative ease of recording DEPT spectra make this spectroscopic technique excellent for deducing the number of ATIII binding sites per chain and, hence, the bioactivity of a LMW heparin. As expected, no correlation could be observed between ATIII binding sites and other activities not dependent on the ATIII binding site, such as, heparin cofactor II activity (not shown).

Capillary electrophoresis was also evaluated as a method for quantifying the ATIII binding sites within LMW heparins. After treatment of a LMW heparin with heparin lyase I, an oligosaccharide map was prepared using capillary electrophoresis (Fig. 4). The area of a peak corresponding to a hexasaccharide **2** containing the 2,3,6-trisulphated glucosamine residue (Fig. 2) found within the porcine mucosal heparin major ATIII pentasaccharide binding site was determined. The mole percent of ATIII binding site for each mole of LMW heparin was calculated assuming an extinction coefficient of 5500 (Rice & Linhardt 1989) for all the oligosaccharides (Table 1). These data are plotted against the anti-FXa activity for each LMW heparin as shown in Fig. 3b. We had previously demonstrated that the concentration of hexasaccharide **2** correlated well with the anti-FXa activity of charge fractionated and affinity fractionated heparins (Kim & Linhardt 1989).

The concentration of hexasaccharide **2** measured by capillary electrophoresis showed no correlation with the anti-FXa activity for LMW heparins prepared by different methods. One possible reason for this may have been our failure to take minor structural variants of the ATIII pentasaccharide binding site into account. A binding site containing a 2,6-disulphated glucosamine flanked by two 2-*O*-sulphated iduronic acid residues (see structure **1**) affords a tetrasaccharide on treatment with heparin lyase I (Loganathan et al 1990). This tetrasaccharide can be seen in the capillary electropherogram as a minor peak (**3**; ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-β-D-GlcAp(1→4)-D-GlcNp2S3S6S (where ΔUAp is 4-deoxy-α-L-*threo*-hex-eno-pyranosyluronic acid and GlcAp is glucopyrano-

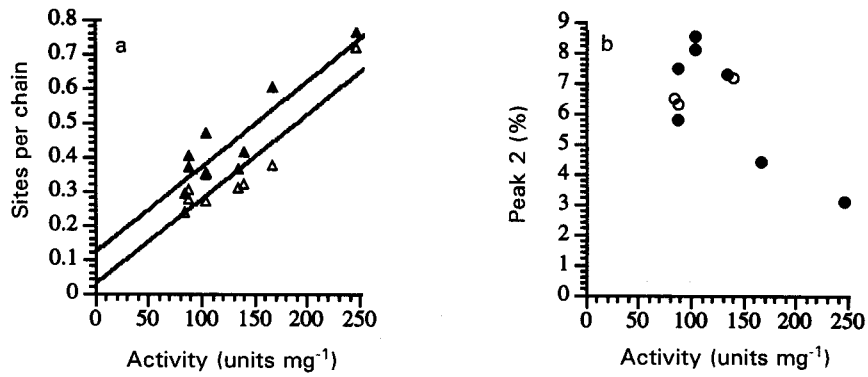

FIG. 3. Correlation of anti-FXa activity. a. ^{13}C NMR: to the number of ATIII binding sites per chain (▲, $r=0{\cdot}82$) and to the ratio of total C-2 signal intensity to C-2 of 3-sulphated glucosamines (△, $r=0{\cdot}84$); b. capillary electrophoresis: to mole percent of hexasaccharide **2** in heparin lyase I digestion mixture of different LMW heparins (●) and different lots of the same LMW heparin (○) ($r=0{\cdot}92$).

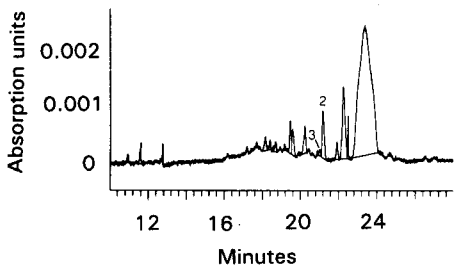

FIG. 4. Capillary electropherogram of the heparin lyase I-treated RD hep-c. The peak at 21·6 min (labelled 2) corresponds to hexasaccharide **2** and the minor peak (labelled 3) corresponds to a tetrasaccharide (see text for structure) arising from a minor structural variant found in porcine heparins ATIII binding site.

syluronic acid) (Loganathan et al 1990). Even if this second minor peak is taken into account, no improvement in the correlation is obtained. A second reason for the poor correlation observed might be an inaccuracy in the measured mass of heparin due to the presence of additives or salt in the pharmaceutical preparations, leading to error in the measured mole % of **2**. A third reason for the poor correlation might be the introduction of structural artifacts in the depolymerization process, making a particular LMW heparin resistant to heparin lyase depolymerization and causing a reduction in the measured content of hexasaccharide **2**. All of these explanations are supported by the observation that different lots of the same LMW heparin preparation, having substantially different anti-FXa activity, gave a reasonable correlation (Fig. 3b).

In conclusion, the use of ^{13}C NMR for determining the proportion of ATIII binding sites within heparin is clearly demonstrated. Capillary electrophoresis is not useful for assessing the activity of different LMW heparins but may be useful in the analysis of different lots of LMW heparin prepared by a single method.

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References

Ampofo, S. A., Wang, H. M., Linhardt, R. J. (1991) Disaccharide compositional analysis of heparin and heparan sulfate using capillary zone electrophoresis. Anal. Biochem. 199: 249–255

Casu, B., Oreste, P., Torri, G., Zopetti, G., Choay, J., Lormeau, J. C., Petitou, M., Sinay, P. (1981) The structure of heparin oligosaccharide fragments with high anti (factor Xa) activity containing the minimal antithrombin III binding sequence. Biochem. J. 197: 599–609

Choay, J., Lormeau, J. C., Petitou, M., Sinay, P., Casu, B., Oreste, P., Torri, G., Gatti, G. (1980) Anti-Xa active heparin oligosaccharides. Thromb. Res. 18: 573–578

Choay, J., Petitou, M., Lormeau, J.C., Sinay, P., Casu, B., Gatti, G. (1983) Structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for ATIII and eliciting high anti-factor Xa activity. Biochem. Biophys. Res. Commun. 116: 492–499

Desai, U. R., Linhardt, R. J. (1994) Molecular weight of low molecular heparins by ^{13}C NMR spectroscopy. Carbohydr. Res. 255: 193–212

Desai, U. R., Linhardt, R. J. (1995) Molecular weight of heparin using ^{13}C nuclear magnetic resonance spectroscopy. J. Pharm. Sci. 84: 212–215

Edens, R. E., Al-Hakim, A., Weiler, J. M., Rethwisch, D. G., Fareed, J., Linhardt, R.J. (1992) Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low-molecular weight heparin derivatives. J. Pharm. Sci. 81: 823–827

Fareed, J., Walenga, J.M., Huan, X., Racanelli, A. (1988) Comparative study on the in vitro and in vivo activities of seven low-molecular-weight heparins. Haemostasis 18a(Suppl. 3): 3–15

Fareed, J., Walenga, J.M., Racanelli, A., Hoppensteadt, D., Huan, X., Messmore, H. (1988) Validity of the newly established low-molecular-weight heparin standard in cross referencing low-molecular-weight heparins. Haemostasis 18a(Suppl. 3): 33–47

Kim, Y. S., Linhardt, R. J. (1989) Structural features of heparin and their effect on heparin cofactor II mediated inhibition of thrombin. Thromb. Res. 53: 55–71

Lindahl, U., Backstrom, G., Thunberg, L. (1983) The antithrombin-binding sequence in heparin. Identification of an essential 6-*O*-sulphate group. J. Biol. Chem. 258: 9826–9830

Linhardt, R. J. (1992) In: Ogura, H., Hasegawa, A., Suami, T. (eds) Carbohydrates—Synthetic Methods and Applications in Medicinal Chemistry. Kodansha/VCH Publishers, Tokyo/ Weinheim pp 385–401

Linhardt, R. J., Loganathan, D, G. (1990) In: Gebelein (ed.) Biomimetic Polymers. Plenum Press, New York, pp 135–175

Linhardt, R. J., Rice, K. G., Merchant, Z. M., Kim, Y. S., Lohse, D. L. (1986) Structure and activity of a unique heparin-derived hexasaccharide. J. Biol. Chem. 261: 14448–14454

Linhardt, R. J., Loganathan, D., Al-Hakim, A., Wang, H. M., Walenga, J. M., Hoppensteadt, D., Fareed, J. (1990) Oligosaccharide mapping of low molecular weight heparins: structural differences and their relationship to activity. J. Med. Chem. 33: 1639–1645

Linhardt, R. J., Wang, H. M., Loganathan, D., Bae, J. H. (1992) Search for the heparin antithrombin III binding site precursor. J. Biol. Chem. 267: 2380–2387

Loganathan, D., Wang, H. M., Mallis, L. M., Linhardt, R. J. (1990) Structural variation in the antithrombin III binding site region and its occurrence in heparin from different sources. Biochemistry 29: 4362–4368

Lohse, D. L., Linhardt, R. J. (1992) Purification and characterization of heparin lyases from *Flavobacterium heparinum*. J. Biol. Chem. 267: 24347–24355

Merchant, Z. M., Kim, Y. S,, Rice, K. G., Linhardt, R. J. (1985) Structure of heparin derived tetrasaccharides. Biochem. J. 229: 369–377

Neville, G. A., Mori, F., Holme, K. R., Perlin, A. S. (1989) Monitoring the purity of pharmaceutical heparin preparations by high-field ^{1}H-nuclear magnetic resonance spectroscopy. J. Pharm. Sci. 78: 101–104

Rice, K. G., Linhardt, R. J. (1989) Study of defined oligosaccharide substrates of heparin and heparan monosulphate lyases. Carbohydr. Res. 190: 219–233

Tachibana, S., Nishiura, S., Ishida, S., Kakehi, K., Honda, S. (1990) Quality check of heparin injections by ^{1}H-nuclear magnetic resonance spectroscopy. Chem. Pharm. Bull. 38: 2503–2506

Walenga, J. M., Bara, L., Samama, M. M., Fareed, J. (1985) Amidolytic antifactor Xa assays in laboratory evaluation of heparin and low molecular weight fractions. Semin. Thromb. Hemost. 11: 100–107

Walenga, J. M., Fareed, J., Hoppensteadt, D., Emanuele, R. M. (1986) In vitro evaluation of heparin fractions: old vs. new methods. CRC Crit. Rev. Clin. Lab. Sci. 22: 361–389