

Theme Issue Article

Structural features of low-molecular-weight heparins affecting their affinity to antithrombin

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Summary

As part of a more extensive investigation on structural features of different low-molecular-weight heparins (LMWHs) that can affect their biological activities, Enoxaparin, Tinzaparin and Dalteparin were characterised with regards to the distribution of different chain length oligosaccharides as determined by size-exclusion (SE) chromatography, as well as their structure as defined by 2D-NMR spectra (HSQC). The three LMWHs were also fractionated into high affinity (HA) and no affinity (NA) pools with regards to their ability to bind antithrombin (AT). The HA fractions were further subfractionated and characterised. For the parent LMWHs and selected fractions, molecular weight parameters were measured using a SE chromatographic system with a triple detector (TDA) to obtain absolute molecular weights. The SE chromatograms clearly indicate that Enoxaparin is consistently richer in shorter oligosaccharides than Tinzaparin

and Dalteparin. Besides providing the content of terminal groups and individual glucosamine and uronic acid residues with different sulfate substituents, the HSQC-NMR spectra permitted us to evaluate and correlate the content of the pentasaccharide, AT-binding sequence A-G-A*-I-A (AT-bs) through quantification of signals of the disaccharide sequence G-A*. Whereas the percent content of HA species is approximately the same for the three LMWHs, substantial differences were observed for the chain distribution of AT-bs as a function of length, with the AT-bs being preferentially contained in the longest chains of each LMWH. The above information will be useful in establishing structure-activity relationships currently under way. This study is therefore critical for establishing correlations between structural features of LMWHs and their AT-mediated anticoagulant activity.

Keywords

Low-molecular-weight heparin, structure, affinity fractionation, size fractionation, 2D-NMR

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Introduction

Low-molecular-weight heparins (LMWHs), are a class of anti-thrombotic drugs derived from unfractionated heparin (UFH), which have been introduced into clinical practice some 20 years ago. Their always increasing use in the treatment of thromboembolic and cardiovascular disorders is justified by a number of distinct advantages that these compounds offer over UFH. Besides minimisation of some unwanted side effects of UFH, such as bleeding, thrombocytopenia (1) and osteoporosis, LMWHs also exhibit a higher bioavailability, prolonged plasma half-life and a more predictable pharmacological response (2–4), together with a reduced anti-factor IIa activity with respect to anti-factor Xa activity (5–7).

Heparin is a linear polymer made up of 1→4 linked disaccharide repeating units, consisting of a α -D-glucosamine (A) and a hexuronic acid, α -L-iduronic (I) or β -D-glucuronic (G) acid. Variations within the disaccharide units occur especially in the form of O-sulfation in position 2 of iduronic acid (I_{2S}), and position 3 and 6 of glucosamine (A_{3S}, A_{6S}) or in the form of N-sulfation or N-acetylation at position 2 of the glucosamine (A_{NS}, A_{NAC}). This array of structural features generates a potential total of 48 different disaccharide combinations; however, not all of these possible combinations are allowed by the biosynthetic process. Different sulfation patterns are unevenly distributed along the heparin chain: the disaccharide sequence –I_{2S}–A_{N,6S}– is the major repeating structural unit and constitutes the highly sulfated region within heparin and are primarily lo-

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cated toward the non-reducing end of the heparin chains. Conversely, undersulfated sequences containing nonsulfated iduronic acid residues and A_{NAC} are prevalently located toward the reducing end of the polymer (8). Some of the heparin chains contain a specific pentasaccharide sequence, $-A_{\text{NAC},6\text{S}}-G-A_{\text{NS},3\text{S},6\text{S}}-I_{2\text{S}}-A_{\text{NS},6\text{S}}-$ (A-G-A*-I-A) constituting the antithrombin (AT) binding site (AT-bs), characterised by a central rare trisulfated glucosamine, $A_{\text{NS},3\text{S},6\text{S}}$ (A*) (9–11).

Ideally, LMWHs should differ from their parent heparins only for their average molecular weight, which are approximately one third of that of the original UFH. In practice, depending on methods used for manufacturing LMWHs, LMWHs differ from their parent heparin not only in terms of molecular weight but also in terms of monosaccharide composition and oligosaccharide sequence. A number of strategies have been designed to obtain LMWHs, including size fractionation of heparin to select lower molecular weight material and partial cleavage of heparin chains through chemical or enzymatic processes. The three most commonly used commercial LMWHs are produced by different methods of depolymerisation, each resulting in unique structural alterations at either the reducing and/or non-reducing end of the cleaved heparin chains (12, 13). Enoxaparin is the result of chemical β -eliminative cleavage by alkaline treatment of heparin benzyl esters that generates $\Delta 4,5$ uronic acid residues at the non-reducing end. For this LMWH also the presence of 1,6-anhydro aminosugars at the reducing end has been described (14). Tinzaparin, prepared through enzymatic β -eliminative cleavage of unmodified heparin by heparinase-I, is also characterised by a unsaturated uronate residue present at the non-reducing end of virtually every chain. Dalteparin is produced through deaminative cleavage with nitrous acid followed by reduction, resulting in formation of an anhydromannitol ring at the reducing end.

Besides the chemical nature of the terminal monosaccharide residue, the internal structure of LMWHs differs from that of parent heparins, depending on the cleavage point along the heparin chain. In particular, preferential cleavage at either highly sulfated regions or the undersulfated ones influences disaccharide sequence distribution (i.e. monosaccharide composition and substitution pattern) of resulting LMWHs. Furthermore, the type of depolymerisation can affect the preservation and location of the specific pentasaccharide sequence AGA*IA of the active site for AT (15, 16). Previous studies have identified the fact that a pentadecasaccharide with the antithrombin binding sequence located at the reducing end is optimal for bridging the AT/FIIa complex (16). Moreover, both the reducing and non-reducing extension of the pentasaccharide, together with possible structural modifications inside the pentasaccharide itself, may sensibly influence the binding ability to AT and consequently affect the biological activity of LMWHs (15, 18). Functional assays performed *in vitro*, evaluating plasma protein binding and AT-mediated antiprotease activity, showed wide variations among the commercially available LMWHs, indicating that their compositional differences have an important impact on biological activity (19).

With the aim of deepening the knowledge of structural features within LMWHs which may correlate with their biological activities, the present work focuses on the investigation of the composition of the three most common commercial LMWH,

Enoxaparin, Tinzaparin and Dalteparin. Each of them was characterised for its affinity to AT and separated into high-affinity (HA) and no-affinity (NA) fractions. Both fractions and the corresponding parent LMWHs were characterised for their molecular weight measured *via* HP-SEC/TDA (20), and for their monosaccharide composition evaluated by two-dimensional nuclear magnetic resonance (2D-NMR) (21). Each HA fraction was further separated into three sub-fractions, with graded affinity towards AT. Importantly, we find that the amount of A* is significantly increased in the HA fractions compared to either the parent LMWH or the NA fraction as is the amount of the disaccharide G-A*, consistent with the known structure of the AT-bs. Furthermore, we find that this latter signature is a more reliable marker of the pentasaccharide motif than A* alone. Finally, comparison of Enoxaparin, Tinzaparin and Dalteparin to one another indicated while there were some structural similarities, there were also substantial molecular differences between the samples.

Materials and methods

Materials

Enoxaparin was supplied by Sanofi-Aventis Pharma (Milan, Italy) as injectable Clexane; Tinzaparin was supplied by LEO-Pharma (Ballerup, Denmark) as a powder; Dalteparin was from Pharmacia AB (Stockholm, Sweden) as injectable Fragmin. Antithrombin was obtained from Kybernin P1000 pharmaceutical preparation (ZLB Behring GmbH, Marburg, Germany).

Size-exclusion chromatography

SE chromatography was performed on Bio-gel P10 column (2.6 cm x 160 cm) as previously described (22).

Affinity chromatography

Two columns of different capacity were prepared for analytical and preparative purposes. 10 mg of AT were coupled to cyanogen bromide (CNBr) activated Sepharose 6 Fast Flow (Pharmacia), as described by Hook et al. (23), to obtain an analytical column (0.9 x 5.5 cm) of AT-Sepharose gel. Following the same procedure, 100 mg of AT was employed to set up a preparative column (1.6 x 10 cm). AT was previously purified by ultrafiltration with centricon YM P1000 (Amicon) starting from Kybernin P1000 (ZLB, Behring GmbH, Marburg, Germany). Preliminary experiments to assess the maximum heparin binding capacity of the AT-Sepharose column were carried out by loading different amounts of Enoxaparin. It was found that for amounts equal to or lower than 2 mg and 15 mg respectively, the relative percentages of interacting species were constant.

Analytical affinity chromatography

Of the samples, 1.5 mg were dissolved in 1.5 ml of equilibrium buffer (Tris-HCl 50 mM pH 7.4, NaCl 50 mM) and loaded onto the column (flow rate 0.5 ml/min). Heparin chains with no affinity toward the antithrombin were eluted with 12 ml of equilibrium buffer and collected as a single fraction called NA fraction. Heparin chains with high affinity toward the antithrombin were eluted with 10 ml of elution buffer (Tris-HCl 50 mM pH 7.4, NaCl 2.5 M) and collected as a single fraction named HA frac-

tion. The column was finally washed with 70 ml of equilibrium buffer. All the effluent fractions from AT-Sepharose column were analysed for uronic acid content by the carbazole reaction (24). In each colorimetric assay, the calibration curve was built up with an amount of the same LMWH solution loaded onto AT-Sepharose column.

Subfractionation of HA fractions

To separate HA fractions into subfractions having graded affinity toward AT, a preparative AT-Sepharose column was overloaded with an amount of LMWH (50 mg) corresponding to three times the retention capacity of the resin. After a first chromatography, performed as described above, buffer at high ionic strength eluted the first HA fraction, named HA1, whereas buffer at lower ionic strength eluted a fraction composed by both heparin chains devoid of affinity for AT and chains exceeding the retention capacity of the resin. This material was loaded again onto the column and eluted under the same conditions as above: HA2 and a second flow-through fraction were obtained. The re-loading procedure was repeated twice again until no HA fraction was retained by the column. A further HA fraction (HA3) and a final fraction depleted of affinity for AT (NA), were obtained. All the fractions were analysed for uronic acid content by the carbazole reaction. Finally, fractions HA and NA were desalted first by ultrafiltration through Amicon chamber (160 ml) with a 500 Da cut off Millipore filter, then by size exclusion chromatography with TSK-gel HW40S column (2.6 x 58 cm). To remove the traces of Tris a cation-exchange chromatography was finally performed on Amberlite IR-120 [H⁺] column (2 x 12.5 cm). Samples were neutralised with 0.1 N NaOH.

Molecular weight evaluation

Molecular weight determinations were performed by HP-SEC-TDA on a Viscotec (Houston, Texas) instrument equipped with a VE1121 pump, Rheodyne valve (100 µl), and triple detector array 302 equipped with RI, viscometer, and light-scattering (90° and 7°) systems. A G2500 and G3000 7.8mm x 30 cm TSK GMPWL Tosoh columns were used with 0.1 M NaNO₃ as eluent (flow, 0.6 ml/min). Samples were dissolved in the eluent solution at 5 mg/ml. Chromatogram analysis was performed with OMNISEC 4.1 software, all molecular weight parameters were obtained, number-average mean molecular weight (M_n), weight-average mean molecular weight (M_w), and polydispersity (M_w/M_n, D), of each sample.

NMR spectra

Spectra were recorded at 25°C on a Bruker Avance 600 spectrometer (Karlsruhe, Germany) equipped with 5-mm TCI cryoprobe. In proton spectra water signal was presaturated during relaxation delay. Samples were prepared by dissolution of 2–10 mg of heparin in 0.6 ml of deuterium oxide. A recycle delay of 10 seconds and from 32 to 128 number of scans were used, depending on the sample amount. Two-dimensional heteronuclear single quantum coherence (2D-HSQC) spectra were recorded with carbon decoupling during acquisition with 320 increments of 32–64 scans for each. The polarisation transfer delay ($D = 1/[2 \cdot {}^1J_{C-H}]$) was set with a ${}^1J_{C-H}$ coupling values of 150 Hz. The matrix size 1K x 512 was zero filled to 4K x 2K by application of a

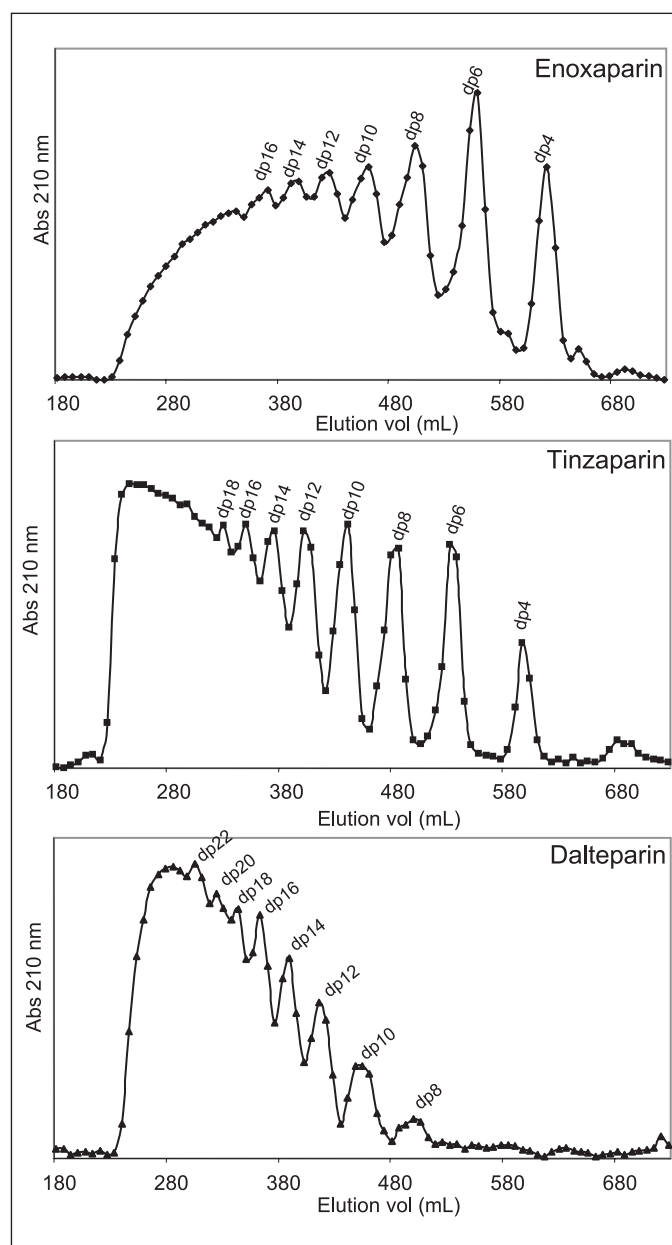


Figure 1: Gel permeation elution profiles on Bio-gel P10 of the three LMWHs studied.

squared cosine function prior to Fourier transformation. Integration of peak volumes was made using standard Bruker TOPSPIN 2.0 software. The monosaccharide composition was calculated following the procedure previously published (13). The average degree of polymerisation (dp) is calculated through the ratio between the total volume of anomeric signals (uronic acids, glucosamines and LR residues) and that of anomeric signal residues at the reducing end (including the first galactose residue of LR at 104.0/4.526 ppm) (25). M_n is obtained by multiplying dp value by the average M_w of disaccharide (calculated by NMR considering the average acetylation and sulfation degree).

Results

Size-homogeneous chromatographic fractions

The three LMWH preparations were separated into their oligomeric components through gel permeation chromatography on Bio-gel P10, obtaining notably different chromatographic profiles (Fig. 1). A series of peaks, each one corresponding to size-homogeneous oligomeric families, ranging in size from dp2 to dp16 (Enoxaparin) or dp18 (Tinzaparin) and from dp8 to dp22 (Dalteparin), were resolved. The larger unresolved oligosaccharides were present in different proportions for each LMWH. The average chain length of most of the components was determined through analysis by mass spectrometry, by collecting the fractions corresponding to individual peaks (data not shown). Moreover, for enoxaparin between peaks of the shortest major oligomers (e.g. tetramers, hexamers etc.) small peaks were observed and were identified as arising from odd-numbered oligomers (i.e. trimer in front of the tetramer; pentamer between the tetramer and hexamer). From this, a first important compositional difference is immediately apparent: the three LMWHs greatly differ in their oligosaccharidic distribution. In particular Enoxaparin is the richest in small oligosaccharidic chains from dp2 to dp8; Dalteparin is the richest in chains larger than dp12, whereas chains under dp8 are not detectable or negligible. Among the three LMWHs, Tinzaparin is the one with the highest polydispersity, all the oligomeric families being represented. Each oligomeric fraction was collected and their relative percentage with respect to the total compound was estimated (Fig. 2).

Affinity chromatography fractions

Affinity chromatography on AT-Sepharose of LMWHs, resulted in separation of two portions: the first, eluting at lower ionic strength, represents the non-adsorbed material and was designated as the “no affinity” (NA) fraction; the second, eluting at higher salt concentration, was designated the “high affinity” (HA) fraction. The relative content of HA fraction of the three LMWHs, calculated as a mean of three experiments, appeared very similar: 13.6 ± 0.71 for Enoxaparin, 14.2 ± 0.62 for Tinzaparin, and 13.6 ± 1.21 for Dalteparin.

With the aim of further fractionating HA components, the AT-Sepharose column was overloaded with an amount of each

LMWH corresponding to three times the retention capacity of the resin. Passage of a large excess of heparin species through the affinity column permits a continuous exchange of adsorbed material and a selection, by multiple passages, of the more strongly interacting species. Three HA subfractions endowed with graded strength of interaction with AT were separated from each LMWH (HA1, HA2 and HA3, in scaling order of affinity), together with the corresponding no affinity fraction (NA).

Molecular weights evaluation

Molecular weight parameters of Enoxaparin, Tinzaparin and Dalteparin, and their corresponding NA and HA fractions, were evaluated by HP-SEC/TDA. HP-SEC on-line with triple detector array permits the evaluation of the size of polymeric species by exploiting the combined and simultaneous action of three detectors: laser light scattering (RALLS/LALLS), refractometer and viscometer. The method, successfully employed to determine the molecular weight of UFH and dermatan sulfate (20), does not require any chromatographic column calibration. A comparison of the elution profile of the three LMWHs together with a typical UFH is displayed in Figure 3. The resulting molecular weight parameters, Mn, Mw and D, are shown in Table 3. In agreement with the diversity of Biogel P10 elution profiles that was observed for the LMWHs (Fig. 1) the parent LMWHs exhibited very different molecular weight and polydispersity values. Tinzaparin, had the highest average Mw (8300 Da) and exhibited the broadest polydispersity (1.40); Dalteparin with an Mw of 6900 Da had the lowest polydispersity degree (1.22), thus appearing as the most homogeneous sample; Enoxaparin had the lowest Mw (5300 Da) and an intermediate D value (1.30). All the HA fractions exhibited significantly higher molecular weight with respect to their parent LMWH and, in the case of Enoxaparin and Tinzaparin, they also had a reduced polydispersity. Of note is the fact that no chains below 2400 Da were detected in the HA fractions.

Of the HA subfractions, only HA1 and NA of all LMWHs and HA3 of Enoxaparin were in sufficient quantity for HP-SEC/TDA analysis. We find that all HA1 fractions exhibited a decreased Mn value and, in parallel, an increase in polydispersity compared to the HA fraction as a whole (Table 1). The HA3 fraction of Enoxaparin showed, in turn, consistently reduced Mn,

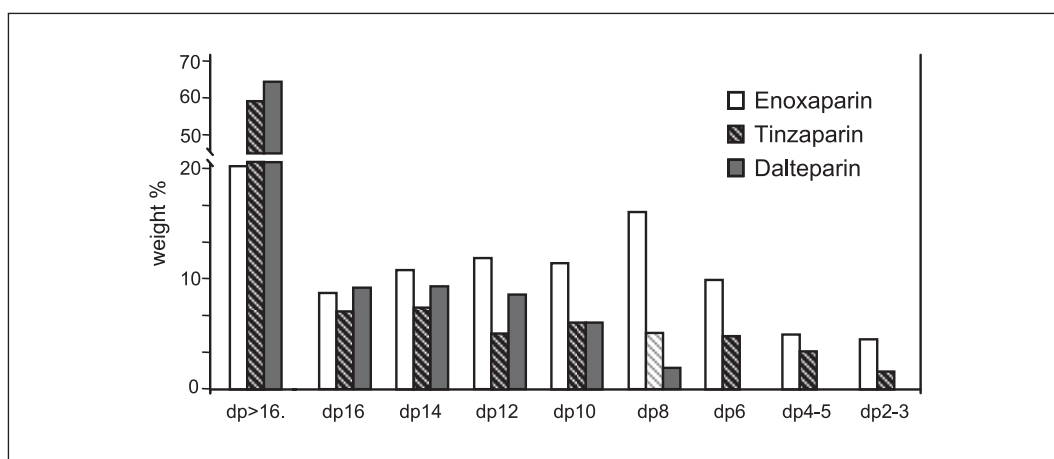
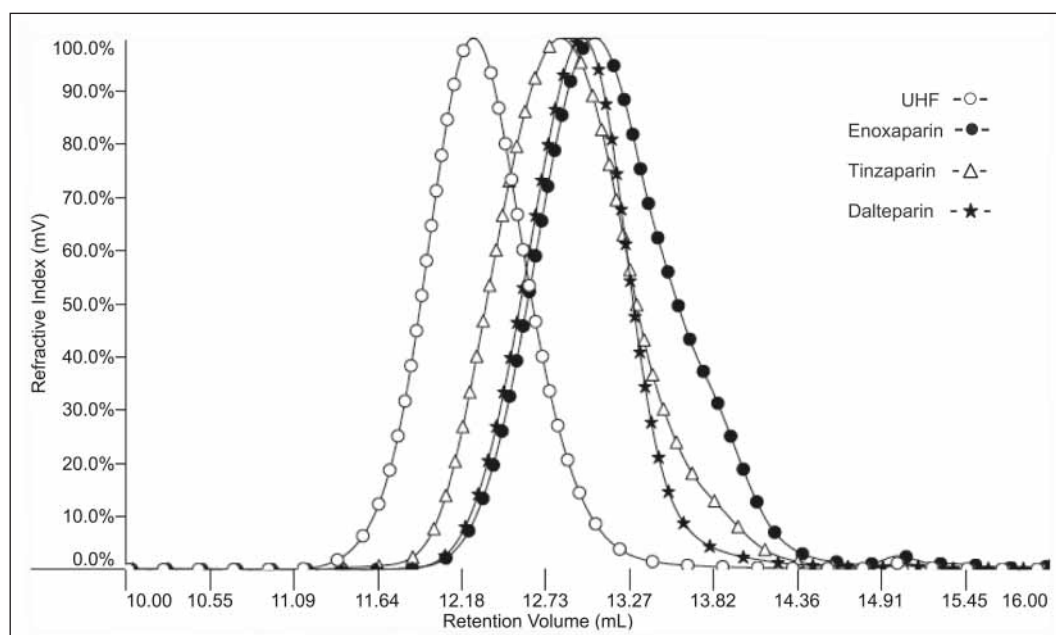


Figure 2: Relative abundance of the oligosaccharidic components of the three LMWHs studied (weight %). All percentages were calculated on the basis of the weight recovery after the Bio-gel P10 column.

Figure 3: HP-SEC/TDA chromatograms (Refractive Index response vs Retention Volume) of the three LMWHs studied in comparison with a typical UHF.



Mw and D values with respect to the corresponding HA fraction. The Mw values of NA fractions obtained after normal and overloading chromatographic conditions respectively, were comparable. In all cases, NA fractions exhibited lower Mw values with respect to parent LMWHs than did the corresponding HA fractions.

NMR characterisation

All fractions and subfractions separated by affinity chromatography were studied using the quantitative compositional analysis method based on two-dimensional (2D) ^1H - ^{13}C correlation measurements (heteronuclear single quantum coherence, HSQC) that have been previously applied towards the characterisation of the corresponding parent LMWHs (13). The average monosaccharide content of the three LMWHs and of all their fractions are presented in Table 2. The data obtained for Enoxaparin, compared to that obtained for Tinzaparin and Dalteparin confirm that Enoxaparin contains a larger number of different monosaccharide residues (Fig. 4). Whereas we have identified that oligosaccharide chains within Enoxaparin and its fractions can have eight distinct reducing residues, Tinzaparin and Dalteparin and their fractions have a lower number of possibilities: i.e., two and one, respectively (13). Slight differences in the monosaccharide composition can be observed between LMWHs (i.e. a higher content of N-acetyl-glucosamine of Tinzaparin with respect to Enoxaparin and Dalteparin), however, the total sulfation degree, calculated by adding all different sulfated monosaccharides, was almost the same (2.4–2.6) for all LMWHs, indicating that lower N-sulfation is compensated for by somewhat higher O-sulfation. Notably, the “linkage region” (LR) reminiscent of the attachment, through a serine (Ser) residue, to the peptide core of the original proteoglycans (26), is appreciable only for Tinzaparin (3–4%) and practically absent in Enoxaparin and Dalteparin ($\leq 1\%$). The observed difference

might be related either to the manufacturing process or to the parent heparin used for the LMWH production, or to both.

Although both A* and G linked to A* [G-(A*)] residues are present within the pentasaccharide motif, as has been demonstrated previously, we find that only G-(A*) reliably correlates with the content of AT-bs. Indeed, weaker signals associated with A* residues are found in heparin fractions with no affinity for

Table 1: Molecular weight parameters determined via HP-SEC/TDA, of the three LMWHs and of their corresponding HA and NA fractions. (n.d.: not determined)

Sample	Fraction	Mn	Mw	D
Enoxaparin	Parent LMWH	3800	5300	1.30
	HA	7300	8400	1.15
	HA1	5600	8800	1.56
	HA3	4800	5900	1.23
	NA	3300	4400	1.30
Tinzaparin	Parent LMWH	5900	8300	1.40
	HA	9500	11100	1.17
	HA1	8400	11000	1.29
	HA3	n.d.	n.d.	n.d.
	NA	5300	7600	1.45
Dalteparin	Parent LMWH	5700	6900	1.22
	HA	7900	9500	1.20
	HA1	6400	8500	1.32
	HA3	n.d.	n.d.	n.d.
	NA	4800	6600	1.35

Table 2: Percent contents of variously substituted glucosamines and uronic acids in different disaccharide sequences of Enoxaparin, Tinzaparin and Dalteparin. Linkage region (LR) content, calculated from the average content of the constituent monosaccharides (GlcA, Gal and Xyl) with respect to the total glucosamine, and the degree of sulfation (DS), calculated adding the molar fractions of each sulfated monosaccharide, are also reported. In italics, the neighbour residues at the reducing side are indicated.

Sample	Glucosamines							LR	Uronic acids								DS	
	<i>A_{NS}</i> <i>I_{2s}</i>	<i>A_{NS}</i> <i>I</i>	<i>A_{NS}</i> <i>G</i>	<i>A_{NS,3S,6S}</i> <i>(A*)</i>	<i>A_{NAC}</i>	residues at reduc- ing end	total <i>A_{6S}</i>		<i>I_{2s}</i>	<i>I</i> <i>A_{6S}</i>	<i>I</i> <i>A_{6OH}</i>	<i>G</i> <i>A_{NS,3S,6S}</i> <i>[G-(A*)]</i>	<i>G</i> <i>A_{NS}</i>	<i>G</i> <i>A_{NAC}</i>	<i>G_{2S}</i>	total Δ <i>U</i>		<i>I_{2s}</i> <i>red</i>
Enoxaparin																		
Parent LMWH	44.4	8.8	14.5	4.4	10.7	17.3	84.2	0.7	49.9	5.7	1.0	3.2	9.5	4.1	2.6	19.1	2.1	2.51
HA	35.3	13.8	10.4	14.0	16.0	10.5	89.5	0.7	49.1	13.8	0	11.2	6.9	3.8	1.5	13.0	0.7	2.48
HA1	33.5	14.6	11.0	16.8	12.5	11.5	90.1	0.7	49.4	12.4	0.7	10.7	6.0	2.3	1.1	17.3	0.2	2.59
HA3	38.1	13.6	8.9	11.5	15.8	12.0	88.7	0.6	49.8	13.4	0	9.7	7.0	3.3	1.7	14.3	0.7	2.48
NA	51.4	5.7	12.1	1.6	8.2	21	81.6	0.6	53.9	3.8	1.3	1.0	9.5	4.3	2.3	22.0	1.8	2.53
Tinzaparin																		
Parent LMWH	55.7	6.9	8.2	2.0	14.6	12.5	81.9	3.1	60.9	6.5	2.1	2.1	9.3	4.4		14.7		2.50
HA	43.4	13.5	8.0	7.9	19.6	7.3	85.6	3.8	56.8	13.1	1.6	6.2	6.4	3.8		11.8		2.42
HA1	40.2	14.3	9.1	10.1	18.7	7.2	87.0	4.6	54.6	13.9	1.9	7.2	6.6	4.5		9.9		2.43
HA3	45.6	13.3	8.6	5.3	20.6	7.2	80.8	4.6	60.7	12.0	1.4	3.2	6.9	3.5		11.8		2.39
NA	54.6	6.2	9.0	0.7	14.0	14.2	85.2	3.2	60.0	4.3	1.9	0.8	10.9	5.1		16.6		2.44
Dalteparin																		
Parent LMWH	56.8	8.0	5.2	4.6	10.3	15.5	91.6	0.6	75.4	9.5	0.5	4.1	6.8	3.6				2.63
HA	45.5	15.0	5.2	12.9	11.9	9.4	89.8	1.1	60.9	14.1	2.3	12.4	6.6	3.8				2.50
HA1	41.8	13.5	8.8	12.9	12.0	10.9	94.1	1.2	65.0	13.5	1.4	12.3	4.8	3.0				2.59
HA3	44.3	14.2	4.7	12.8	12.8	11.2	91.7	1.0	66.5	14.7	0.8	10.6	4.6	2.8				2.57
NA	65.8	5.3	5.6	1.7	6.3	14.1	91.8	0.7	82.4	5.1	0.7	0.8	6.8	4.2				2.65

AT, indicating that this residue can be located also in sequences not directly involved in the interaction with AT (27, 28). Conversely, the amount of G-(A*) found in the three LMWHs follows the same trend as their reported anti-Xa activity, increasing from Tinzaparin (2.1%, 90 UI/mg), to Enoxaparin (3.2%, 104 UI/mg), to Dalteparin (4.1%, 122 UI/mg) (29). In Figure 5, the G-A* disaccharide is highlighted within the structure formula of AT-bs.

Nevertheless, the most important differences are displayed by each HA and NA fraction with respect to the corresponding parent LMWH. All signals of residues associated to the pentasaccharide sequence are stronger in the HA and especially in the HA1 fractions with respect to the parent LMWHs and very weak in the NA fractions (Table 2 and Fig. 4). The amount of both A* and G-(A*) increases about three times in HA fractions with respect to the parent LMWH. A parallel increase is also observed for signals associated to iduronic acid linked to N-sulfated-6-O-sulfated glucosamine [I-(A_{6S})], N-acetylated glucosamine (A_{NAC}) and N-sulfated glucosamine linked to non-sulfated iduronic acid residues [A_{NS}-(I)]. The latter residues are not exclusive to the pentasaccharide sequence AGA*IA, since they are

present also in other positions of the heparin chains (12, 27). However, A_{NAC} (or less frequently A_{NS}) is the first residue of the pentasaccharide and invariably precedes the natural AT-binding domains of heparin and HS.

Less pronounced differences were observed between the HA1 and HA3 fractions of the same LMWH. A slight decrease of G-(A*) content from the HA1 to the HA3 fractions of Enoxaparin and Dalteparin was observed, while the content of the other residues related to the pentasaccharide sequence varies within experimental error. Only in Tinzaparin is there a decrease of about 50% of G-(A*) and A* going from the HA1 (7.2 %) to the HA3 (3.1 %) fraction.

The average Mn values obtained via NMR (Table 3) and via HP-SEC/TDA (Table 1) exhibit the same trend of variation within each family of fractions, confirming the higher molecular weight of the HA fraction with respect to the NA fractions and the corresponding parent LMWH. With regards to the differences between the HA1 and HA3 fractions of Enoxaparin and Dalteparin, three different situations were observed. In particular, i) for Enoxaparin, a slight decrease of Mn with a dp difference of 2–3 monosaccharidic units, ii) for Tinzaparin, a slight in-

Table 3: Average Mn and degree of polymerisation (dp) evaluated via HSQC-NMR together with the estimated G-(A*) content per chain.

Sample	Fraction	Mn	dp	G-(A*)/chain
Enoxaparin	Parent LMWH	3200	10.5	0.2
	HA	6500	20.0	1.1
	HA1	5900	18.5	1.0
	HA3	5100	16.0	0.8
	NA	3000	9.5	0
Tinzaparin	Parent LMWH	4100	13.0	0.2
	HA	6600	21.0	0.7
	HA1	5900	19.0	0.7
	HA3	6600	21	0.4
	NA	3900	12.0	0
Dalteparin	Parent LMWH	4500	14.0	0.3
	HA	6200	20.0	1.2
	HA1	5800	18.5	1.1
	HA3	5900	18.5	1.0

crease of AT-bs content per chain from HA1 to HA3 appears to be responsible for their different affinity to AT, structures additional and/or alternative to the AT-bs are needed to explain the apparently low content of A-G-A*-I-A in the HA species of Tinzaparin. More in depth investigations are required to clarify this point.

Discussion and conclusions

Although the internal structure of LMWH chains is largely unaffected by the most common depolymerisation processes, different approaches to the cleavage of the parent heparin dictates the type of terminal groups for a particular LMWH, as well as the average size of fragments, and dispersion of molecular weights around the average values (12, 28). A first level of characterisation of LMWHs accordingly involves identification and quantification of terminal groups (13) and determination of the average molecular weight and polydispersity. A second, important

level requires determination of the AT-binding sequences (AT-bs). In the present study, assessment of the content of AT-bs was approached both by affinity chromatography which determines the percent content of species with HA for AT (9), and by quantification of NMR of signals identified as specific for the disaccharide G-A* prevalently present in the AT-bs (13).

Besides generating different terminal residues, different methods of depolymerisation of heparin currently used to generate LMWHs may cleave glycosidic bonds both outside and inside the AT-bs. Despite any expected process-related differences, the percent content of HA species determined by affinity chromatography was quite similar for Enoxaparin, Tinzaparin, and Dalteparin. However, such a content is not a direct measure of the actual content of AT-bs when this sequence is contained in chains of different length. As illustrated in Figures 1, 2 and 3, the average MW and the distribution of different oligosaccharide components is quite different for the three LMWHs. As shown in Table 1, such a difference is amplified when comparison is made between the HA and NA fractions of each LMWH, HA chains being consistently longer than NA chains. It is noteworthy that our data indicates that no chains under 2400 Da were detected in HA fractions. Although a detailed comparison of compositional data for the three LMWHs is outside the scope of this publication, it is worth noting that the LR is contained in significant amounts (3.1%) only in Tinzaparin and that it is distributed in all its fractions regardless of their affinity for AT. More relevant to the main aim of this study is the comparison of contents of A* and G-A* components (shaded columns in Table 2). Both residues may cause overestimation the active sequence A-G-A*-I-A, especially when this sequence lacks the essential 6-O-sulfate group at residue A at its non-reducing end. Accordingly, low but not negligible amounts of both A* and G-A* components were detected also in NA fractions.

The most relevant difference emerging from comparison of the NMR spectra of the three series of LMWH derived fractions is the content of AT-bs per chain (Table 3) estimated by NMR from the marker signal G-(A*) [as proposed by Guerrini et al. (13)]. We find that the level of G-(A*) is quite different for individual LMWHs as well as for the HA, HA1, HA3 and NA fractions of each of them.

Furthermore, the data presented in Table 3 deserves additional comment. First, the content of G-A* per average chain of Enoxaparin and Dalteparin is slightly higher than 1 (1.1 and 1.2, respectively), suggesting that some of the chains of these

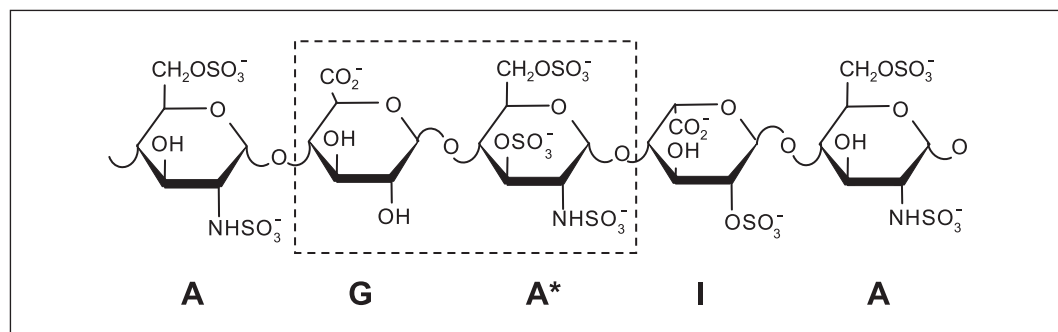


Figure 5: Structure of the pentasaccharide sequence AGA*IA constituting the antithrombin binding site (AT-bs). The G-A* disaccharide is highlighted in the dashed frame.

LMWHs, especially the longest chains, may contain two AT-bs, as already observed for UFH (27). On the other hand, data for the HA fraction of Tinzaparin and for its subfractions are unexpectedly low (0.7 and 0.4, respectively). Although these data may be explained by structures contributing to the apparent affinity for AT which do not contain intact A-G-A*-I-A sequences, this hypothesis requires in depth studies to be validated.

In this sense, in addition to the different content of AT-bs of heparin oligosaccharides, the extension of the active pentasaccharide sequence can also influence the AT binding properties of an oligosaccharide chain and regulate its interaction with factor IIa as well. This, in turn, may influence the anti-factor Xa and anti-factor IIa activities of the three LMWHs. Since the AT-bs can occur randomly within HA heparin chains, its differential positioning, together with the occurrence of glucuronic acid rather than iduronic acid at the non-reducing end of AGA*IA (14), can affect the strength of interaction with AT and, possibly,

anti-Xa activity. Moreover, when the extension of the AT-bs toward the non-reducing end is sufficiently long (at least 10 residues) to accommodate thrombin, a tertiary complex can be formed which results in thrombin inhibition (17). The larger the extension of the heparin chain beyond the non-reducing end of AT-bs, the higher is the probability that thrombin molecules can interact with AT (16).

Studies are in progress on the detailed structure of HA and NA fractions of size homogeneous fractions of the three heparins, with the aim of rationalising the contribution of typical LMWH chains to the measured anti-Xa potency and other biological activities.

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