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Low molecular weight heparins: Structural differentiation by spectroscopic and multivariate approaches

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ABSTRACT

Various branded low molecular weight heparins (LMWHs) have been used for the treatment and prevention of thrombotic for over 20 years. With the introduction of generic LMWHs and the recent events involving heparin contamination, a great deal of effort is being expended in investigating ways of monitoring and regulating this class of complex drugs. In this paper, we present the characterization of different forms of LMWHs, as well as the comparison of 5 enoxaparin copies from different manufactures. The data suggests that, while some of these drugs are structurally comparable, specific analytical methods as well as biological and pharmacological tests may be used to address their similarity, quality and potential interchangeability. The proposed approach may also be useful in comparing biosimilar and branded LMWHs.

1. Introduction

Numerous pharmacological actions of heparin are associated with the treatment of thromboembolic diseases. Heparin plays a role in coagulation due to its interaction with blood soluble proteins as well as its interactions with the blood vessel and associated cells. Consequently, blood clot formation can be efficiently moderated by heparin and its derivatives. Unfractionated heparin (UFH) is mostly obtained from porcine and bovine mucosa and has been widely used for the treatment and prevention of thrombotic events. It consists of molecular chains of various lengths varying from 2000 to 40,000 Da (Nader, Lopes, Rocha, Santos, & Dietrich, 2004).

Low molecular weight heparins (LMWHs) are smaller chains of UFH that are obtained by various chemical and enzymatic depolymerization processes. Since they are produced from natural heparins, they must share structural features with the parent compound, however, the depolymerization process used for their production leads to unique structural features.

The main advantages of LMWHs over UFH are improved bioavailability and higher anti-factor Xa/anti-factor IIa activity ratios, with decreased hemorrhagic risk during prolonged treatments (Hoppensteadt, 2006). Lovenox (INN enoxaparin), which is obtained by the β-eliminative cleavage of the heparin benzyl ester by alkaline treatment, is the most popular product with an on growing net global sales rising by over 20% in 2008 (Gray & Mulloy, 2009). Since Lovenox is no longer protected by US patents and with the introduction of generic products in many countries, including the United States, copies of enoxaparin and other branded LMWHs made by different manufactures are being introduced into the market (Ofosu, 2011).

The introduction of biosimilar products may be beneficial since it could result in lower treatment costs. Despite this advantage, they raise new concerns regarding the presence of inactive, uncharacterized, less and/or more active moieties not found in the originator products as well as contaminants (Harenberg et al., 2009). Thus, specific guidelines for the approval of LMWH copies are necessary.

In the present paper, we have examined the chemical characteristics of different LMWHs using a combination of spectroscopic methods and multivariate analysis. This approach provides a simpler and reliable method for establishing the similarity or differences among LMWHs.

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2. Materials and methods

2.1. Heparin and low molecular weight heparins

Enoxaparins (~100 IU/mg) were obtained from Sanofi-aventis Farmacêutica Ltda (Suzano, Brazil), Sanofi-aventis (Bridgewater, USA), Eurofarma Laboratórios Ltda (São Paulo, Brazil), Blausiegel Farmacêutica (Cotia, Brazil), Aspen Pharma (Rio de Janeiro, Brazil) and Laboratório Cristália (Itapira, Brazil); Nadroparins (~110 IU/mg) were obtained from different manufactures; Gammaparin was obtained from Corcon Pharmaceutical, Milan, Italy; Tinzaparin (~90 IU/mg) was obtained from the US market and UFHs (~180 IU/mg) were obtained from Gentium SpA (Villa Guardia (CO), Italy) and Kim Master (Kim Master Produtos Químicos Ltd., Brazil). The glycosaminoglycans (GAGs) – chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S) and dermatan sulfate (DS) – were purchased from Seikagaku Kojii Corporation (Tokyo, Japan). All of the tested agents were in their sodium form.

The enoxaparin copies used for this study received market approval in Brazil and possess anti-factor Xa and IIa activity of 100–110 IU/mg and 25–30 IU/mg, respectively.

2.2. Nuclear magnetic resonance (NMR)

For NMR experiments, the samples were deuterium exchanged by repeated dissolution in D_2O and freeze-drying. Spectra were obtained from solutions in D_2O at 30 °C, using TMSP as standard ($\delta = 0$). All spectra were obtained with a Bruker 400 MHz AVANCE III NMR spectrometer (Bruker Daltonics, Germany) with a 5 mm inverse gradient probe. 1D and 2D assignments were performed using ^{1}H-(zg, and zgpr) and HSQC (hsqcetgpsi) programs. HSQC was acquired using 8–16 scans, respectively, per series of 2 K × 512 W data points with zero filling in F1 (4 K) prior to Fourier transformation (Viccini et al., 2009).

2.3. Scanning UV spectroscopy

Scanning UV spectroscopy was performed on Perkin-Elmer Lambda 25 UV/VIS spectrometer (Turku, Finland). UV spectra were recorded at 1 mg/mL in water from 190 to 320 nm at a scanning rate of 120 nm/min with 1 nm resolution at room temperature.

2.4. Circular dichroism (CD)

CD spectra were recorded on a J-810 spectropolarimeter (JASCO, Easton, MD), using a quartz sample cell of 0.1 cm path length, between 260 and 190 nm at a scanning rate of 50 nm/min with 0.5 nm resolution at 37 °C. The values are presented in Molar CD. CD spectra were recorded at 10 mg/mL in water and are relative to (+)-10-camphorsulfonic acid (1.0 mg/mL). The resulting spectra were saved as ASC files for subsequent multivariate analysis.

2.5. Multivariate analysis

Multivariate analysis was performed as previously described (Lima et al., 2011) using the software R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Viena, Austria. http://cran.r-project.org/), with prior mean centering.

3. Results

3.1. Scanning UV of different LMWHs

The electronic transitions of the carboxylate groups of the uronate residues and the *N*-acetyl chromophores of the hexosamine residues ($n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions) along the GAG chain are the structural features responsible for the majority of the UV spectral bands shown in Fig. 1.

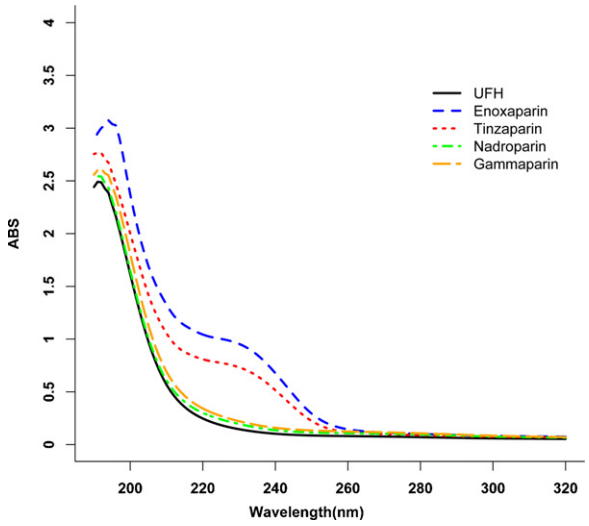

Fig. 1. Scanning UV spectra of different LMWHs. UFH, unfractionated heparin.

The band around 190–200 nm arises mainly from the carboxylate chromophore of iduronate and glucuronate as well as *N*-acetyl chromophores of the hexosamine. For the LMWHs produced by β-eliminative methods, enoxaparins and tinzaparin, an extra band from 215 to 255 nm is observed due to the 4,5 double bond (C=C) present in the non-reducing uronic acid (ΔU).

3.2. CD spectra of LMWHs and GAGs

CD spectra of LMWHs are shown in Fig. 2. The spectral features represent differences in right and left hand circularly polarized light arising from transitions occurring within the oxygen atoms (ring, glycosidic linkage and hydroxyl), maxima around 200 nm, as well as electronic transition occurring within the carboxylate and *N*-acetyl chromophores, negative band with its maxima around 210 nm. Both chemical (enoxaparin) and enzymatic (tinzaparin) β-eliminative methods introduce a 4,5 double bond (C=C) into the uronate residue at the non-reducing end, generating a new chromophore as evidenced by the negative band with its maxima around 230 nm. Interestingly, the CD spectra from LWMHs produced by nitrous acid degradation (nadroparin) and γ-radiation (gammaparin) are comparable to the UFH one, suggesting that no new chromophores were introduced into these molecules throughout their depolymerization process.

It is well known that circular dichroism is sensitive to sugar conformation, since their uronic acids (β-D-Glucuronic acid and α-L-Iduronic acid) give rise to signals of opposite sign (Morris, Rees, Sanderson, & Thom, 1975; Rudd et al., 2009). As shown in Fig. 2, the CD spectra for DS, C4S and C6S–GAGs that contain mainly glucuronic acid as their hexuronic acid – are quite peculiar displaying only a broad negative band with its maxima around 210 nm. DS has its negative band slightly shifted with its maxima around 207 nm, which may be explained by the fact that it also contains iduronic acid. Also, the lack of the positive band around 190 nm for DS, C4S and C6S may reflect their difference in glycosidic linkage. The latter compounds are composed of *N*-acetylated β-D-galactosamine (β-linkage), while in heparins the hexosamine moiety is either *N*-acetylated or *N*-sulfated α-D-glucosamine (α-linkage).

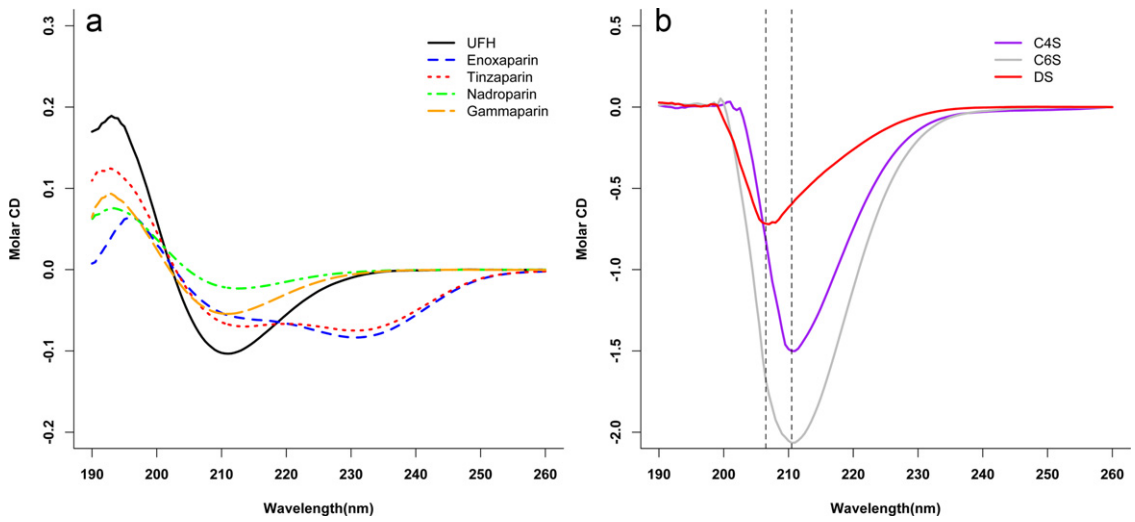

Fig. 2. CD spectra of different LMWHs and natural occurring GAGs. (a) LMWHs and UFH. (b) natural occurring GAGs. UFH, unfractionated heparin; C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; DS, dermatan sulfate. The vertical dashed lines highlight the difference on the negative maxima on DS, C4S and C6S spectra.

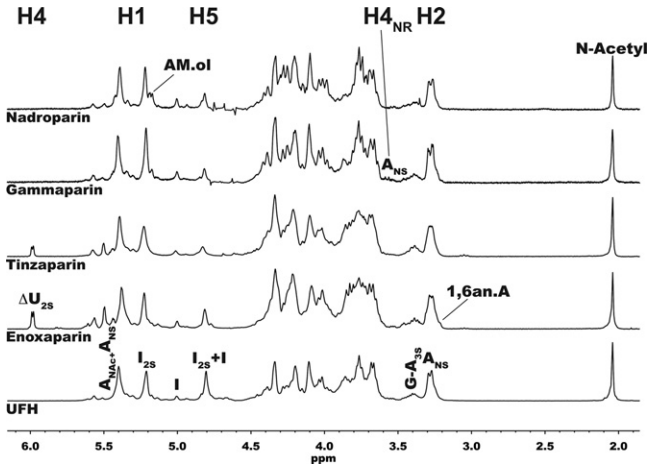

Fig. 3. ^{1}H NMR of UFH and different LMWHs. Major signals are identified. UFH, unfractionated heparin; LMWHs, low molecular weight heparins; A_{NS}, 2-deoxy-2-sulfoamino-D-glucopyranose; I_{2S}, 2-O-sulfo-iduronic acid; G, glucuronic acid; A_{3S}, 2-deoxy-3-O-sulfo-2-amino-D-glucopyranose; A_{NAc}, 2-deoxy-2-acetylamino-D-glucopyranose; 1,6-an.A, 2-amino-1,6-anhydro-2-deoxy-β-D-glucopyranose; AM.ol, 2,5-anhydromannitol; ΔU_{2S}, 2-O-sulfo-4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid, NR, non-reducing end.

3.3. ^{1}H NMR of LMWHs

The major disaccharide repeating unit: -4)-α-L-IdoA2SO$_3$-α-(1→4)-D-GlcNSO$_3$,6SO$_3$ (I_{2S}-$A_{NS,6S}$) present in UFHs corresponds to the major signals on the LMWHs ^{1}H NMR spectra (Fig. 3). Minor signals are due to under- and over-sulfated sequences and those associated with the depolymerization process used in their production.

Enoxaparin and tinzaparin, which are produced by chemical and enzymatic βeliminative reactions, respectively, possess unsaturated 2-O-sulfated uronic acid (ΔU_{2S}) at the nonreducing end of their chains, which is rapidly noticed by a signal around 6 ppm (Fig. 3). Also, the signals around 5.8 pmm and 3.2 ppm, corresponding to ΔU and 2-sulfo-amino-1,6-anhydro-2-deoxy-β-D-glucopyranose (1,6-an.A), respectively, make the enoxaparin ^{1}H NMR spectrum more distinct from heparin than the tinzaparin one as the enzymatic procedure used for the production of tinzaparin does not yield these types of monosaccharides. Moreover, the higher complexity on the enoxaparin spectrum is due to the multi-step reactions and side reactions that occur when its fragments are generated during the alkaline treatment (Guerrini, Guglieri, Naggi, Sasisekharan, & Torri, 2007).

The main difference on Nadroparin ^{1}H NMR spectrum, which is produced by a deamination process where heparin is nitrosylated at the amino group of its *N*-sulfoglucosamine residues (Guerrini et al., 2007), to the UFH spectrum is the signal around 5.2 ppm. This signal represents the 2,5-anhydromannitol (AM.ol) residue which is formed by the rearrangement of the *N*-nitrosulfonamide residues that generates a carbocation at C2, leading to a ring contraction and hydrolysis of the adjacent glycosidic bond producing an anhydromannose residue in the reducing end of the chain, which is further stabilized by reduction with sodium borohydride (Lormeau, 1998).

Gammaparin, a LMWH produced by the physical depolymerization of heparin in aqueous solution in the presence of isopropanol by γ-irradiation (Bisio et al., 2001), has a quite similar ^{1}H NMR spectrum to UFH; the main difference is the increase of signal intensity at 3.56 ppm arising from the H4 of the non-reducing *N*,6-sulfated glucosamine residue (Bisio et al., 2001).

3.4. Monosaccharide composition of LMWHs

Throughout the depolymerization reaction new structures are generated including extra features to the mono-dimensional NMR spectra resulting in stronger signal overlap. For this reason, signals for monosaccharide composition were chosen from those with minimal overlap in the HSQC spectra. Signals corresponding to monosaccharides present in the UFH structure and those from LMWHs were chosen as previously described (Guerrini et al., 2007; Guerrini, Naggi, Guglieri, Santarsiero, & Torri, 2005) and are shown in Fig. 4.

The monosaccharide composition for UFH and LMWH preparations is shown in Table 1. The major difference among the LMWHs arise from monosaccharides unique to each depolymerization process employed and the amount of glucuronic acid linked to $A_{NS,3S}$ ($G\text{-}_{ANS,3S}$), sequence which has only been detected in the pentasaccharide motif active for AT (Guerrini et al., 2007).

3.5. ^{1}H NMR features and monosaccharide composition of 5 enoxaparin copies

Since enoxaparin is the most widely used LMWH form, the comparison among 5 different brands present within the same market was conducted. As anticipated, the major signals on the

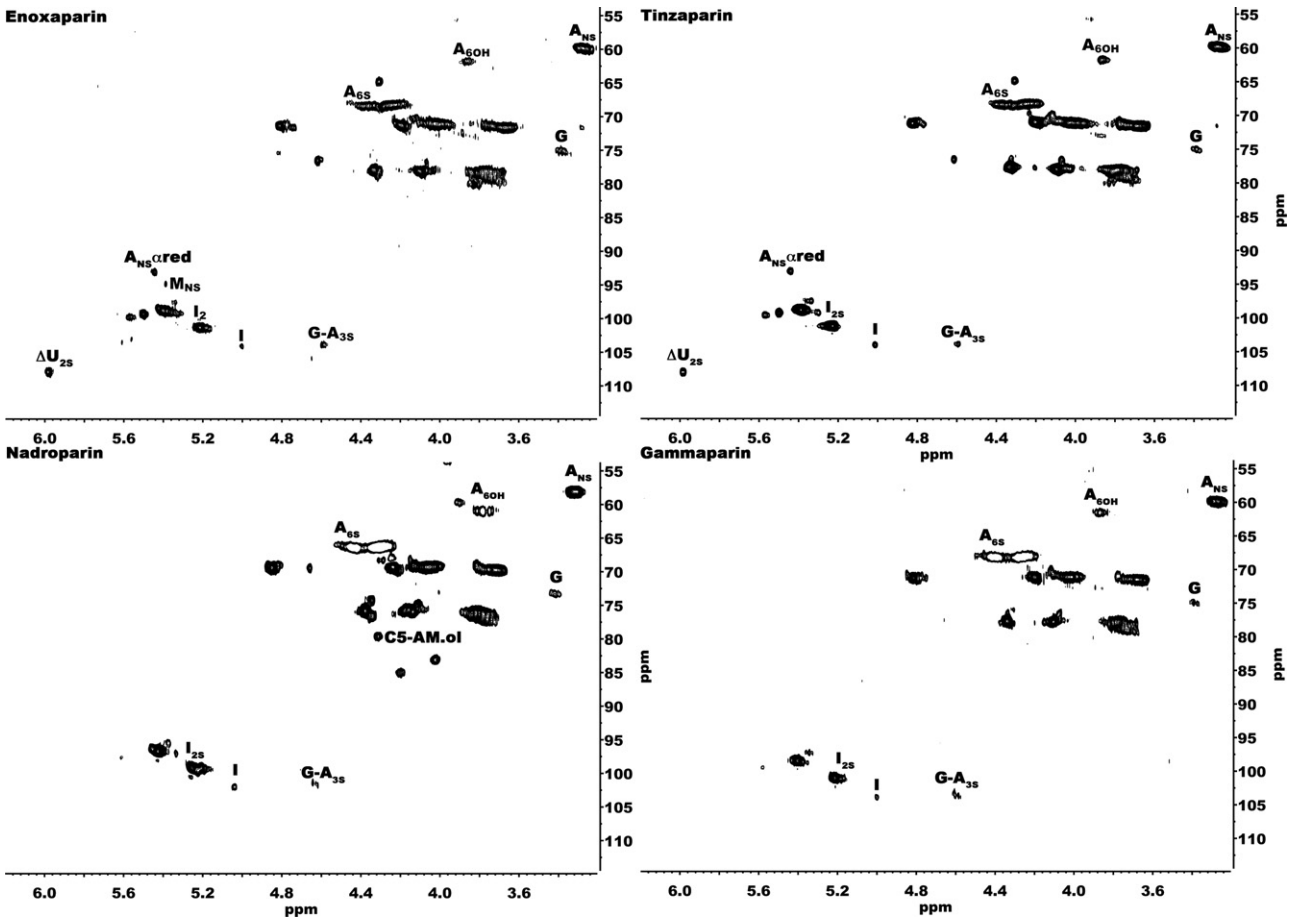

Fig. 4. HSQC spectra of different LMWHs. Signals used for monosaccharide composition are identified. LMWHs, low molecular weight heparins; A_{NS}, 2-deoxy-2-sulfoamino-D-glucopyranose; I_{2S}, 2-O-sulfo-iduronic acid; G, glucuronic acid; A_{3S}, 2-deoxy-3-O-sulfo-2-amino-D-glucopyranose; A_{NAc}, 2-deoxy-2-acetylamino-D-glucopyranose; αred, terminal reducing residue with a configuration; M_{NS}, 2-deoxy-2-sulfamino-D-mannopyranose; 1,6-an.A, 2-amino-1,6-anhydro-2-deoxy-β-D-glucopyranose; 1,6-an.M, 2-amino-1,6-anhydro-2-deoxy-β-D-mannopyranose; AM.ol, 2,5-anhydromannitol; ΔU_{2S}, 2-O-sulfo-4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid; U, Δ4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid.

Table 1
Monosaccharide composition of LMWHs.

Agent	Percentage (%)														Degree of sulfation
	A_{NS}	A_{NAc}	G-$A_{NS,3S}$	A_{6S}	Aα_{NS}red	M_{NS}	1,6 an.A	1,6 an.M	AM.ol	G	I	I_{2S}	ΔU	ΔU_{2S}	
Enoxaparin	75.00	5.50	4.50	90.64	9.00	0.75	2.25	3.00	0.00	14.45	4.62	62.43	0.58	17.92	2.60
Tinzaparin	77.32	5.67	6.96	87.88	10.05	0.00	0.00	0.00	0.00	10.98	4.88	71.95	0.00	12.20	2.66
Nadroparin	73.53	6.62	4.41	92.46	0.00	0.00	0.00	0.00	15.44	13.99	6.29	79.72	0.00	0.00	2.65
Gammaparin	86.46	6.63	6.92	87.24	0.00	0.00	0.00	0.00	0.00	16.48	6.59	76.92	0.00	0.00	2.57

A_{NS}, 2-deoxy-2-sulfoamino-D-glucopyranose; I_{2S}, 2-O-sulfo-iduronic acid; G, glucuronic acid; A_{3S}, 2-deoxy-3-O-sulfo-2-amino-D-glucopyranose; A_{NAc}, 2-deoxy-2-acetylamino-D-glucopyranose; αred, terminal reducing residue with a configuration; M_{NS}, 2-deoxy-2-sulfamino-D-mannopyranose; 1,6-an.A, 2-amino-1,6-anhydro-2-deoxy-β-D-glucopyranose; 1,6-an.M, 2-amino-1,6-anhydro-2-deoxy-β-D-mannopyranose; AM.ol, 2,5-anhydromannitol; ΔU_{2S}, 2-O-sulfo-4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid; U, Δ4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid.

^{1}H NMR spectra corresponded to the trisulfated disaccharide, which is the prevalent repeating unit present on UFH and LMWHs (Fig. 5).

The ^{1}H spectra for all 5 enoxaparins are essentially the same, suggesting that the samples are highly similar. Indeed, as shown by the monosaccharide composition (Table 2), they are quite comparable both in terms of monosaccharides unchanged by the depolymerization reaction and those that arise from it. The similar composition of the five enoxaparins in Table 2 is reflected in their similar anti-factor Xa (100–110 IU) and anti-thrombin (25–30 IU) per mg.

3.6. CD combined with multivariate analysis differentiates LMWHs, UFH and other GAGs

NMR spectroscopy is, perhaps, the most advanced and accepted technique for the differentiation of LMWHs and other complex carbohydrates. However, owing its cost and limited availability, this technique becomes unavailable to many laboratories. Further analysis of the CD spectra performed by multivariate analysis showed that this simple approach could be used as a facile technique for the differentiation among the different classes of LMWHs and GAGs, as well as among the

Table 2
Monosaccharide compostion of enoxaparin copies.

Agent	Percentage (%)												Degree of sulfation
	A_{NS}	A_{NAc}	G-$A_{NS,3S}$	A_{6S}	Aα_{NS}red	M_{NS}	1,6 an.A	1,6 an.M	G	I	I_{2S}	ΔU	ΔU_{2S}	
Enoxa1	74.26	4.95	3.71	90.05	10.40	1.49	1.49	3.71	13.45	3.51	63.16	1.17	18.71	2.61
Enoxa2	72.64	5.57	4.36	86.73	10.17	1.45	2.18	3.63	17.24	3.45	59.77	~1	18.97	2.54
Enoxa3	78.74	5.51	3.15	92.82	9.45	~1	~1	1.57	14.81	3.09	63.58	~1	17.90	2.66
Enoxa4	73.71	4.18	2.95	91.08	14.74	2.21	~1	1.47	12.83	4.28	65.78	~1	16.58	2.67
Enoxa5	75.00	5.50	4.50	90.64	9.00	~1	2.25	3.00	14.45	4.62	62.43	~1	17.92	2.60
Lovenox	76.34	4.58	4.58	92.55	8.4	2.29	1.53	2.29	12.23	4.32	61.15	~1	21.58	2.66

A_{NS}, 2-deoxy-2-sulfoamino-D-glucopyranose; I_{2S}, 2-O-sulfo-iduronic acid; G, glucuronic acid; A_{3S}, 2-deoxy-3-O-sulfo-2-amino-D-glucopyranose; A_{NAc}, 2-deoxy-2-acetylamino-D-glucopyranose; αred, terminal reducing residue with a configuration; M_{NS}, 2-deoxy-2- sulfamino-D-mannopyranose; 1,6-an.A, 2-amino-1,6-anhydro-2-deoxy-β-D-glucopyranose; 1,6-an.M, 2-amino-1,6-anhydro-2-deoxy-β-D-mannopyranose; ΔU_{2S}, 2-O-sulfo-4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid; U, Δ4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid.

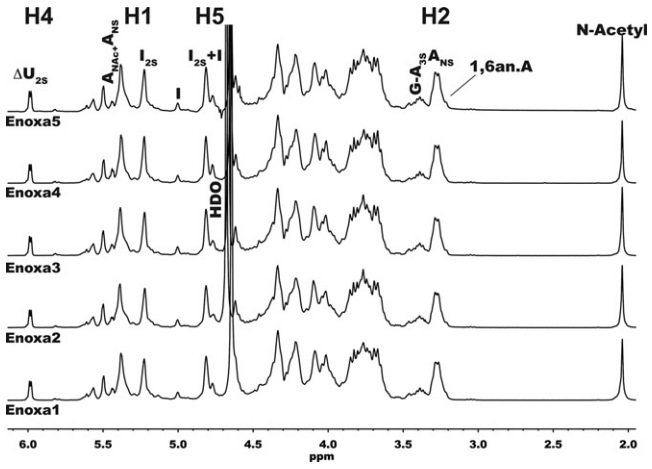

Fig. 5. ^{1}H NMR of generic enoxaparins present on the same market. A_{NS}, 2-deoxy-2-sulfoamino-D-glucopyranose; I_{2S}, 2-O-sulfo-iduronic acid; G, glucuronic acid; A_{3S}, 2-deoxy-3-O-sulfo-2-amino-D-glucopyranose; A_{NAc}, 2-deoxy-2-acetylamino-D-glucopyranose; 1,6-an.A, 2-amino-1,6-anhydro-2-deoxy-β-D-glucopyranose; ΔU_{2S}, 2-O-sulfo-4-deoxy-α-L-threo-hex-4-enopyranosil uronic acid.

same kind of heparin preparation from different manufactures (Fig. 6a–f).

The structural features that are introduced into LMWHs through their unique depolymerization reaction used for their production, as well as differences in the glycosidic linkage, *N*-acetylation, *N*- and *O*-sulfation patterns, monosaccharide composition, different content of hexuronic and hexosamine residues on heparins and naturally occurring GAGs resulted on unique features in their respective CD spectra, which were readily differentiated by circular dichroism combined with multivariate analysis.

4. Discussion

The LMWHs are now globally regarded as drug of choice for post-surgical prophylaxis of deep venous thrombosis (DVT) and, some of them, for the management of acute coronary syndromes. Recently, these agents have also been approved for the treatment of thrombotic disorders such as the cancer related thrombosis and arterial fibrillation.

They are defined as salts of sulfated GAGs having an average MW of less than 8 kDa and for which at least 60% of all molecules have a MW of less than 8 kDa (Pharmacopoeia, 2008), and are produced by fractionation of UFH or by its depolymerization. None of the current LMWH products are prepared by fractionation, though early work used fractions rather than depolymerized fragments (Johnson et al., 1976). For this reason, depending on the patented depolymerization procedure, different products are formed and each one of them has to be regarded as a distinct drug entity, since they exhibit distinct chemical, pharmacological and biomedical profiles (Hirsh, Warkentin, Raschke, Granger, Ohman, & Dalen, 1998).

With the development of various LMWHs, the introduction of generic products in many countries, and the increasing trend to substitute UFH, the need to investigate more deeply the differences among this highly complex kind of pharmacological agent is acquiring importance (Hirsh et al., 1998; Hoppensteadt, Jeske, Fareed, & Bermes, 1995).

Owing their complexity, the differentiation of several forms of LMWHs was performed by spectroscopic and multivariate approaches in order to better understand their different chemical features that might explain their distinct biological/pharmacological activities.

In fact, significant differences were observed regarding their monosaccharide composition, especially the amount of the G-$A_{NS,3S}$, sequence which has been only detected on the pentasaccharide motif active for AT (Guerrini et al., 2007), besides the differences arising from their unique production process.

The difference on the amount of G-$A_{NS,3S}$ directly reflects the fact that the AT binding site can be modified by the depolymerization reactions (Vismara et al., 2010), with a consequent decrease of AT-mediated activity (Fareed, Hoppensteadt, Jeske, Clarizio, & Walenga, 1997). In fact, while a mild nitrous acid treatment, production of Nadroparin, preserve the structural integrity the AT biding motif, strong reaction conditions can generate fragments ending with a trisulfated anhydromannose residue (Casu et al., 1981). Also, enzymatic treatment with heparinases I and II is able to cleave the AT binding site leaving the $A_{N,3,6S}$ as reducing terminal residue (Shriver et al., 2000; Yu et al., 2000). Thus, the reaction procedure used to produce LMWHs with different reactivity towards least sterically hindered regions of the heparin chain can affect the integrity of the AT biding motif (Shriver et al., 2000; Viskov, Just, Laux, Mourier, & Lorenz, 2009; Vismara et al., 2010; Yu et al., 2000).

Differently, gammaparin is produced by a physical method based on controlled gamma irradiation of heparin. A correlation between the amount of irradiation to which heparin is exposed and the reduction of the fragment average size, as well as a good correlation between USP potency and extent of irradiation, has been observed (Bisio et al., 2001). Our data showed that the radiation received by this given sample did not affect the integrity of its AT biding motif which is crucial for a good USP potency.

Enoxaparins are the most common used form of LMWHs. For this reason, a comparison among 5 enoxaparins from different manufactures present within the same market was performed. Their highly correlated monosaccharide composition leads to the conclusion that the starting material used to produce the low molecular mass form probably comes from similar or, perhaps, the same source. Also, the highly similar monosaccharide composition of those related to the depolymerization reaction suggests that different producers used comparable manufacturing conditions. Again,

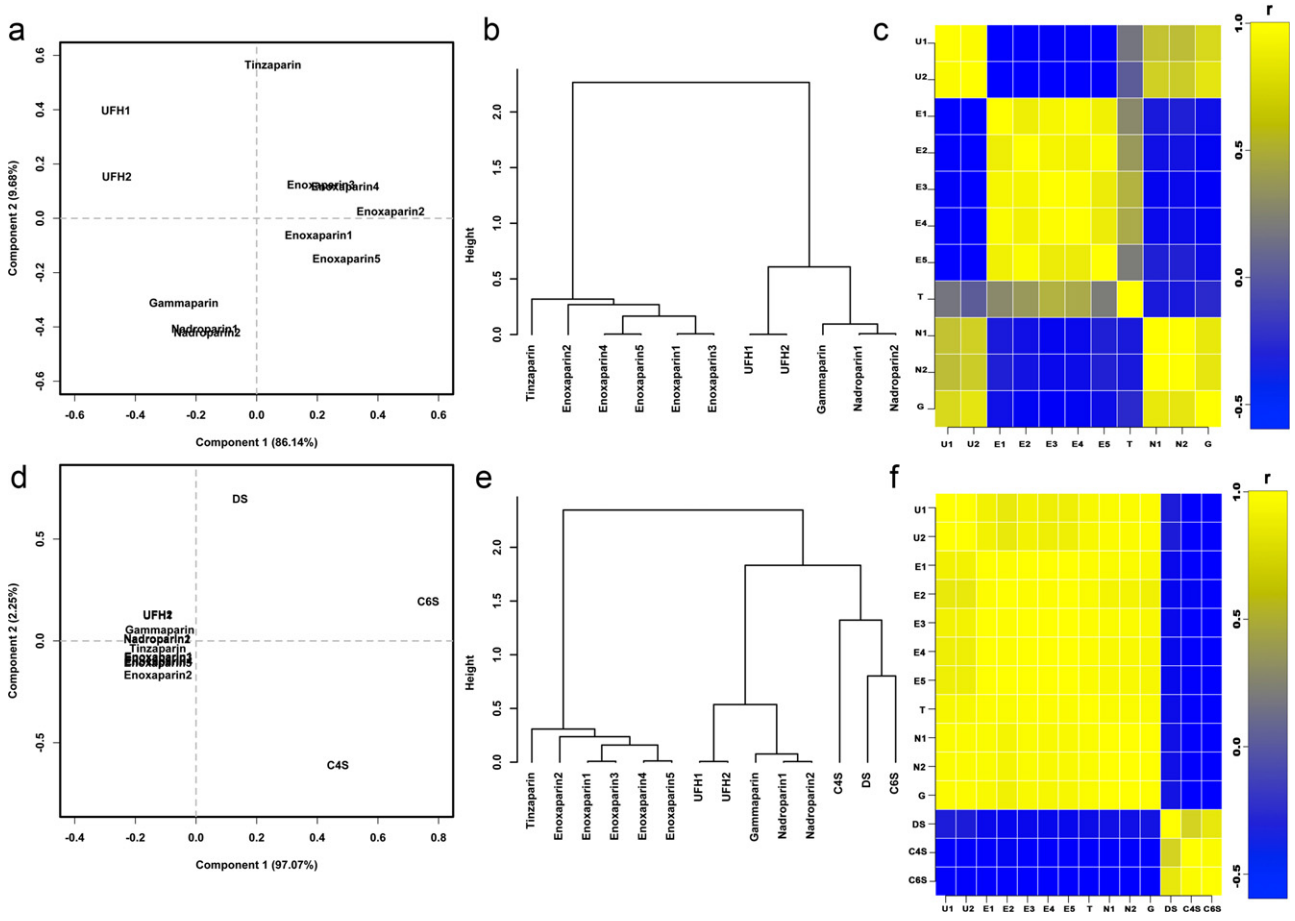

Fig. 6. Multivariate analysis of CD spectra from different LMWHs and naturally occurring GAGs. (a and d) Loading plot. (b and e) Cluster analysis of loading plot. (c and f) Correlation matrix color map. U, unfracionated heparin; E, enoxaparins; T, tinzaparin; N, nadroparin; G, gammaparin; DS, dermatan sulfate; C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; *r*, correlation coefficient.

these data reinforce the idea that the starting material and depolymerization conditions used for the production of LMWHs critically define the final product.

As stated before, the differentiation of LMWHs is quite important, but the most advanced and accepted technique to this matter is NMR, which is also a quite expensive and of limited accessibility technique. Recently, the combination of CD with PCA was used for this matter (Rudd et al., 2009); however, the CD measurements were made on a purpose-built synchrotron CD beamline, which is also of limited access. In this present paper, we used a bench top CD instrument combined with multivariate analysis and similar results were obtained, proving that simple and cheaper approaches can be used to differentiate this class of drugs.

Taken together, the data presented here explain some differences among LMWHs that could lead to their different biological/pharmacological activities. However, further studies are required to better understand the correlation between the structure and biological properties of such drugs. Also, we showed that less expensive spectroscopic methods combined with multivariate approaches may be used as an additional analytical tool for the differentiation of LMWHs and naturally occurring glycosaminoglycans.

As the copy versions of different branded LMWHs may be produced by different manufactures, it is important to characterize these agents by additional methods. The methods outlined in this manuscript represent an approach that could be useful in assessing the structural and chemical equivalence of the newly developed copy versions of branded products.

5. Conflict of interest statement

None declared.

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