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Ultrasonic-assisted preparation of a low molecular weight heparin (LMWH) with anticoagulant activity

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ABSTRACT

Low molecular weight heparin (LMWH) is currently used as an anticoagulant agent and constitutes an alternative to unfractionated heparin, which is the cause of serious adverse drug reaction such as heparin-induced thrombocytopenia (HIT). Commercially available LMWH is produced by enzymatic depolymerization that is costly or by chemical methods that are generally carried out under conditions that could imply side reactions that reduce final product efficiency and yields. In this work, we present the use of a physicochemical method for the production of LMWH. This method consists in the use of hydrogen peroxide-catalyzed radical hydrolysis assisted by ultrasonic waves. LMWH that are produced using this physicochemical method have an average molecular weight and anticoagulant properties (Anti-Xa and Anti-IIa) that are comparable to some of commercial LMWH that are currently used. Ultrasonic-assisted radical depolymerization of heparin leads to products with a remarkably low polydispersity index. Moreover, in comparison to other LMWH such as those produced by enzymatic β-elimination, this physicochemical depolymerization of heparin induces fewer oligosaccharides with less than five monosaccharide units. This contributes to the better preservation of the ATIII pentasaccharide binding sequence, which results in a high Anti-Xa/Anti-IIa ratio (1.86). However, LMWH obtained using this physicochemical method have a lower degree of sulfation than other LMWH, which seems to be the cause of a lower Anti-Xa and Anti-IIa activity (143.62 ± 5.42 and 77.07 ± 4.4, respectively).

1. Introduction

Heparin is a complex acidic polysaccharide that belongs to a class of compounds known as glycosaminoglycans (GAGs) that are found on the cell surface and in the extracellular matrix. Heparin precursor is characterized by a linear chain of a disaccharide unit of *N*-acetyl-D-glucosamine that is bound to D-glucuronic acid (GlcNAcα1–4GlcAβ1–4). The disaccharide repeat unit can be modified to include N- and O-sulfation (6-*O*, 3-*O* and 2-*N* sulfation of the glucosamine and 2-*O* sulfation of the uronic acid) and epimerization of β-D-glucuronic acid to α-L-iduronic acid (Sasisekharan & Venkataraman, 2000). These variations result in a very heterogeneous molecule. Unfractionated heparin (UFH) has an average molecular weight (MW_{avg}) of 15,000 Da (Sommers et al., 2011). Low-molecular-weight heparin (LMWH, $MW_{avg} \geq \sim 6000$) and ultra-low-molecular-weight heparin (ULMWH, $MW_{avg} < 2000$) are heparin derivatives (Hao, Zhang, Yu, Cheng, & Ji, 2011).

Heparin is found in mast cell granules complexed with a number of basic proteases. The anticoagulant and antithrombotic activity of heparin is prevalently due to the specific antithrombin-binding pentasaccharide sequence where the central glucosamine is not only 2- and 6-O-sulfated but also 3-O-sulfated (Mulloy, Hogwood, & Gray, 2010). Therefore, heparin and its derivatives are widely used in the prevention and treatment of thrombosis. However, UFH has low bioavailability (Emanuele & Fareed, 1987) and can cause some serious adverse drug reaction such as heparin-induced thrombocytopenia (HIT) (Warkentin et al., 1995) and a risk of bleeding (Da Silva & Sobel, 2002). The depolymerization of heparin produces smaller molecules (LMWH and ULMWH) that have a better bioavailability and a lower risk of adverse drug reaction.

LMWH can be prepared either using chemical methods with peroxide hydrogen oxidative depolymerization (ardeparin sodium,

Abbreviations: LMWH, low molecular weight heparin; HIT, heparin-induced thrombocytopenia; GAGs, glycosaminoglycans; UFH, unfractionated heparin; MW_{avg}, average molecular weight; ULMWH, ultra-low-molecular-weight heparin; SEC, size exclusion chromatography; M_n, number-averaged molecular weight; M_w, weight-averaged molecular weight; DP, degree of polymerization; *I*, polydispersity index.

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Normiflo®), deaminative cleavage with nitrous acid (dalteparin sodium, Fragmine®), β-eliminative cleavage by alkaline treatment (enoxaparin sodium, Lovenox®) or enzymatic methods using heparinase (tinzaparin, Innohep®) (Linhardt & Gunay, 1999). As enzymatic methods are costly, chemical methods are carried out under strong conditions involving many side reactions that reduce the efficiency of the final product and the yields (Linhardt & Gunay, 1999). Therefore, development of a new method is required for safe, costless and easy preparation of LMWH.

Ultrasonic depolymerization has been investigated with various polysaccharides such as xanthan (Milas, Rinaudo, & Tinland, 1986), starch and chitosan (Czechowska-Biskup, Rokita, Lotfy, Ulanski, & Rosiak, 2005). Commonly, these macromolecules exposed to high-energy ultrasound show permanent reductions in solution viscosity or gel strength due to decreases in molecular weight and its distribution (Lii, Chen, Yeh, & Lai, 1999). This decrease is attributed to the breakage of covalent bonds in the polymer chain. Moreover, the ultrasonic depolymerization of polysaccharides has been shown to completely stop when a minimum molecular mass is reached (Lorimer, Mason, Cuthbert, & Brookfield, 1995; Miyazaki, Yomota, & Okada, 2001). The use of ultrasonic waves can be combined with other depolymerization methods such as radical depolymerization (Petit, Noiret, Guezennec, Gondrexon, & Colliec-Jouault, 2007).

Tinzaparin is a LMWH produced by enzymatic depolymerization using the heparin lyase (heparinase) from *Flavobacterium heparinum* (Mousa, 2002). Three heparinases (I, II, and III) have been isolated from *F. heparinum*. These enzymes cleave sulfated polysaccharide chains containing 1–4 linkages between hexosamines and O-sulfated uronic acid residues *via* a β-elimination mechanism (Korir & Larive, 2008). The reaction yields oligosaccharide products (mainly disaccharides) containing unsaturated uronic acids. Heparinase I (EC 4.2.2.7) selectively cleaves highly sulfated polysaccharide chains which bind hexosamines to 2-O-sulfated α-L-idopyranosyluronic acid residues.

In this work, our aim was to produce LMWH using a physicochemical method involving hydrogen peroxide-catalyzed radical hydrolysis assisted by ultrasonic waves. The average molecular weight, the degree of sulfation and the anticoagulant activities (anti-Xa and anti-IIa) of the physicochemical depolymerization products were measured and compared to commercial LMWH.

2. Experimental

2.1. Materials

All reagents, unless otherwise specified, were purchased from Sigma Aldrich.

Porcine mucosal heparin sodium salt (167 U/mg LOT 9695F) was obtained from MP Biomedicals. Heparin unit (U) is defined as the anticoagulant properties of a given heparin product as it acts on antithrombin III (AT-III). *Flavobacterium* heparinase I (10 IU/mL) was purchased from IBEX. One heparinase international unit (IU) is defined as the amount of enzyme that will liberate 1.0 μmol of unsaturated oligosaccharides from porcine mucosal heparin per minute at 30 °C and pH 7.0. Nadroparin (Fraxiparine®; 7600 IU Anti-Xa/0.8 mL), enoxaparin (Lovenox®; 8000 IU Anti-Xa/0.8 mL), tinzaparin (Innohep®; 14,000 IU Anti-Xa/0.7 mL) and dalteparin (Fragmine®; 2500 IU Anti-Xa/0.2 mL) were kindly provided by the hospital of La Rochelle, France.

Anti-thrombin, factor Xa, factor IIa and chromomeric substrates (CBS 31.39 and CBS 61.50) were purchased from Diagnostica Stago (France) as part of the Stachrom® ATIII kit and the Stachrom® HEPARIN kit.

2.2. Enzymatic depolymerization of unfractionated heparin by heparinase I

Heparin was dissolved to a final concentration of 25 mg/mL of heparin (5 mL) in a reaction mixture containing 0.2 M NaCl, 0.02% BSA and 5 mM Na_2HPO_4. Heparinase I was added to a final concentration of 0.05 IU/mL and the reaction was conducted at 30 °C in a Radleys® reactor under stirring at 500 rpm. A zerotime aliquot (250 μl) was removed from the solution prior to enzyme addition. Aliquots were taken over time (0 h, 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 5 h, 8 h, 24 h, 28 h, 32 h, 48 h) and the reaction was each time stopped by boiling for 5 min, followed by a 5 min centrifugation at 18,000 × g.

2.3. Physicochemical depolymerization of unfractionated heparin by ultrasonic-assisted radical depolymerization

Heparin was dissolved in water to a final concentration of 25 mg/mL (5 mL). The reaction mixture was sonicated using a probe-type UP50H ultrasonicator provided by Hielscher (Germany). The ultrasonicator was equipped with a probe (micro tip MS3) with a diameter of 3 mm, which provided an amplitude of 180 μm. The processor generated mechanical longitudinal vibrations with a frequency of 30 kHz that were produced by electrical excitation. The reaction mixture was maintained at 60 °C in a Radleys® reactor and ultrasonic waves were applied as a 0.5 s pulse to prevent mixture heating. A zerotime aliquot (250 μl) was removed from the solution prior to reaction launching by simultaneous addition of hydrogen peroxide and application of ultrasonic waves. Hydrogen peroxide was added to obtain a final hydrogen peroxide/heparin (w/w) ratio of 0.15 (3.75 mg/mL). The reaction was also conducted without ultrasonication or hydrogen peroxide.

2.4. SEC-HPLC analysis

Structural and quantitative analyses of reaction products by size exclusion chromatography (SEC) were conducted using a LC/MS-ES system from Agilent (1100 LC/MSD Trap VL mass spectrometer) with one TSK-GEL G3000SW and two TSK-GEL G2000SW columns mounted in series. The columns were maintained at 30 °C and the products were eluted with 0.1 M ammonium acetate at a flow rate of 1 mL/min. The products were detected and quantified by differential refractometry using HP Chemstation software off-line for the processing.

Heparin oligosaccharides of different molecular weights purchased from Iduron (Manchester, UK) were used as calibrants for the standard curve.

Number-averaged molecular weight (M_n), weight-averaged molecular weight (M_w), degree of polymerization (DP) and polydispersity index (I) were calculated as follows (Ye et al., 2009):

$$M_n = \frac{\sum N_i \times M_i}{\sum N_i}$$

$$M_w = \frac{\sum N_i \times M_i^2}{\sum N_i \times M_i}$$

$$DP = \frac{M_n}{M_0}$$

$$I = \frac{M_w}{M_n}$$

where N_i is the number of moles of polymer species, M_i the molecular weight of polymer species and M_0 is the molecular weight of monomer unit.

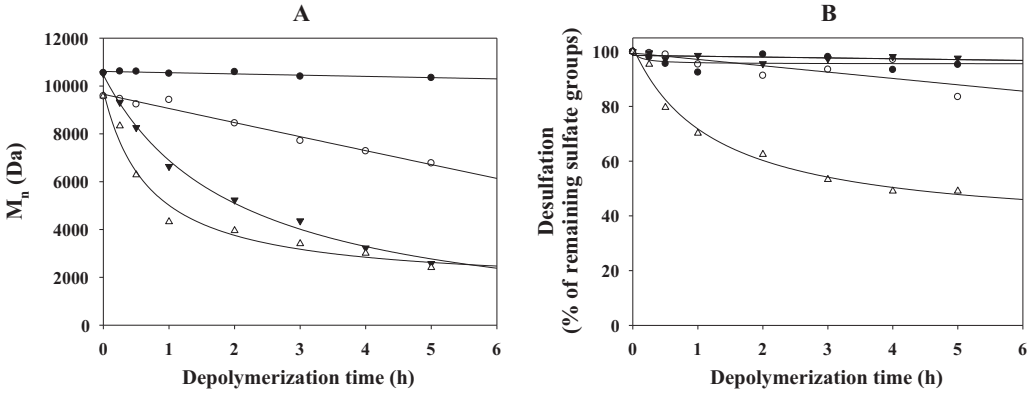

Fig. 1. Effect of the depolymerization methods on the molecular weight (A) and the desulfation (B) of heparin. Ultrasonic waves alone (●), 3.75 mg/mL hydrogen peroxide (○), 0.05 IU/mL heparinase I (▼) and ultrasonic waves coupled with 3.75 mg/mL hydrogen peroxide (△).

2.5. Sulfate determination

The stability of the sulfate groups on the sugar backbone was monitored using 3-amino-7-(dimethylamino) phenothizin-5-ium chloride (Azure A), which binds the sulfated groups in a polysaccharide chain (Gao, Jiao, Ding, & Chen, 2003; Jaques, Ballieux, Dietrich, & Kavanagh, 1968). 2 mL of a 10 mg/L solution of aqueous Azure A were added to 200 μL of a 1000-fold diluted sample of heparin (25 mg/L). Absorbance was then measured at 535 nm.

2.6. Anticoagulation activity of heparin products

Heparin products (6.25 ng/μL) and anti-thrombin III (0.125 μg/μL for anti-Xa assay and 0.625 μg/μL for anti-IIa assay) were incubated at 37 °C in 96-well plates for 2 min. Then, factor Xa or factor IIa were added at a final concentration of 11.25 nKat/mL and incubated for 30 s before the addition of 3.25 nM of the factor Xa chromogenic substrate (CBS 31.39; CH_2SO_2-D-Leu-Gly-Arg-pNA, AcOH) for anti-Xa assay or 1.4 nM of the factor IIa chromogenic substrate (CBS 61.50; EtM-SPro-Arg-pNA, AcOH). The reaction mixture was incubated for 5 min and the absorbance was read at 405 nm every 10 s (Javot, Lecompte, Rabiskova, & Maincent, 2009). The initial velocity was calculated as the slope of the linear segment of the kinetics curve. Anti-Xa and Anti-IIa activity was calculated using a standard curve of different concentrations of unfractionated heparin (0.1–2 U/mL).

3. Results and discussion

The unfractionated heparin starting material was characterized by a number average molecular weight of 11 kDa and a percent of sulfation of 17% (weight of sulfate group/weight of heparin), which corresponds to a degree of sulfation of approximately one sulfate group for every two monosaccharide residues.

Unfractionated heparin was subjected to depolymerization under different chemical, enzymatic and physicochemical conditions. Fig. 1 shows the effect of depolymerization methods on the number-averaged molecular weight (A) and the desulfation (B) of heparin.

In a first time, the depolymerization of heparin using *Flavobacterium* heparinase I was carried out as model reaction of LMWH production, using 0.05 U/mL of enzyme. The number average molecular weight decreased from 11,000 Da to about 2000 Da (DP ≈ 9) during the first 5 h of the reaction and the thermodynamic equilibrium seemed to be reached after 6 h. As expected, no desulfation of the heparin polysaccharide chain was observed: after 6 h of enzymatic depolymerization, 100% of the sulfate groups remained on the side backbone of the depolymerized heparin products.

In a second time, our aim was to produce LMWH with a physicochemical method for the depolymerization of heparin, involving radical-catalyzed hydrolysis using hydrogen peroxide, assisted by ultrasonic waves. The first observation is that ultrasonic waves alone affected neither the molecular weight of heparin nor the sulfate groups on its saccharide backbone. This observation is not surprising considering that the literature clearly shows that ultrasonic waves usually induce the depolymerization of big polymers but stop when a minimal size is reached, depending on the nature of the polymer and the operating conditions. This minimal value has been identified, for example, as 10,000 Da for hyaluronic acid, which is an unsulfated glycosaminoglycan that is constituted by a repeating disaccharide unit of *N*-acetyl-D-glucosamine that is bound to D-glucuronic acid (Miyazaki et al., 2001). The heparin starting material was characterized by an average molecular weight of about 11,000 Da, which was probably lower than the minimal size required to observe depolymerization under the experimental conditions used.

The use of 3.25 mg/mL hydrogen peroxide alone induced the radical depolymerization of heparin, leading to a number-averaged molecular weight of 7500 Da (DP ≈ 34). Hydrogen peroxide affected sulfate groups and provoked a slight desulfation, with more than 80% of sulfate groups retained on the polysaccharide backbones. Hydrogen peroxide has already been used for the oxidative depolymerization of heparin to produce ardeparin sodium (Normiflo®, Wyeth-Ayerst, USA). This drug was withdrawn from the U.S. market in March of 2000 but it is noteworthy that the Food and Drug Administration specified it was not withdrawn for reasons of safety or effectiveness (FDA, 2002). Hydrogen peroxide is also used for the oxidative depolymerization of heparin with Cu^+ to produce parnaparin sodium, commercialized under the trade name Fluxum® by Alfa Wassermann in Italy (Cosmi & Palareti, 2012).

The combination of hydrogen peroxide with ultrasonic waves resulted in a faster radical depolymerization of heparin than the use of hydrogen peroxide alone. After 5 h of reaction, the polymeric fraction had a number-averaged molecular weight approximately equal to 2500 Da, corresponding to a DP of about 9 saccharide units. On the other hand, the desulfation rate was shown to increase and led to the loss of about 50% of sulfate groups after 5 h of reaction. Previous studies that described the depolymerization of a bacterial exopolysaccharide by ultrasonication showed that the combination of ultrasonic waves and hydrogen peroxide enhance the efficacy of depolymerization (Petit et al., 2007).

Table 1
Molecular weight of unfractionated heparin (UFH), commercial LMWH and LMWH produced by depolymerization catalyzed by radical hydrolysis, assisted (ultrasound + H_2O_2 after 1 h) or not (H_2O_2 after 5 h) by ultrasonic waves, or by heparinase I (after 8 h).

	M_n (Da)	M_w (Da)	DP	Polydispersity index	Degree of sulfation
UFH	10,518 ± 616	11,685 ± 522	44	1.11 ± 0.017	1.13
LMWH (ultrasound + H_2O_2)	4324 ± 270	5985 ± 369	18	1.38 ± 0.011	0.7
LMWH (H_2O_2)	6778 ± 189	8484 ± 206	28	1.25 ± 0.053	0.9
Heparinase I	2282 ± 127	7486 ± 248	9.5	3.28 ± 0.075	1.10
Nadroparin (Fraxiparine®)	4598 ± 247	5207 ± 227	19	1.13 ± 0.021	1.48
Dalteparin (Fragmine®)	5835 ± 271	6626 ± 222	24.5	1.13 ± 0.016	1.5
Tinzaparin (Innohep®)	5833 ± 223	7333 ± 155	24.5	1.25 ± 0.028	1.24
Enoxaparin (Lovenox®)	4372 ± 231	5629 ± 233	18	1.28 ± 0.034	1.19

To better understand the size distribution of heparin depolymerization products after ultrasonic-assisted radical depolymerization, size exclusion chromatography (SEC) was performed.

Fig. 2 shows the SEC analysis of the heparin standards. To our knowledge, this is the first time that the use of three columns mounted in series is described. This method enhances the resolution of separation to such an extent that the interval between the elution times of two heparin molecules exhibiting only a difference of two monosaccharide units is longer than 0.5 min.

Fig. 3 shows the SEC analysis of crude heparin depolymerization products (A) and commercial LMWH (B).

After 8 h, heparinase I-catalyzed depolymerization gave rise to oligosaccharide products that in the majority had an even number of saccharide units. This is due to the selective enzymatic β-elimination mechanism. In fact, heparinase I cleaves the highly sulfated heparin chain between the hexosamine and the 2-O-sulfated α-L-iduronic acid residues, resulting mainly in even saccharides containing unsaturated uronic acids at their non-reducing end. We also noted that a part of the unfractionated heparin was still present in the reaction medium and was thus not depolymerized after 8 h of reaction.

The chromatogram corresponding to the products obtained using ultrasonic-assisted radical depolymerization after 1 h of reaction showed a lower resolution than that obtained for the heparinase I-catalyzed depolymerization. This suggests that these products have a more heterogeneous structure, with oligosaccharides that have both odd and even numbers of saccharide units (Bisio et al., 2004). This physicochemical method of heparin depolymerization is thus not selective and seems to be caused by a mechanism of random scission of glycosidic linkages. In fact, the degradation of macromolecules provoked by ultrasound treatment is mainly attributed to cavitation effects (Grönroos et al., 2001).

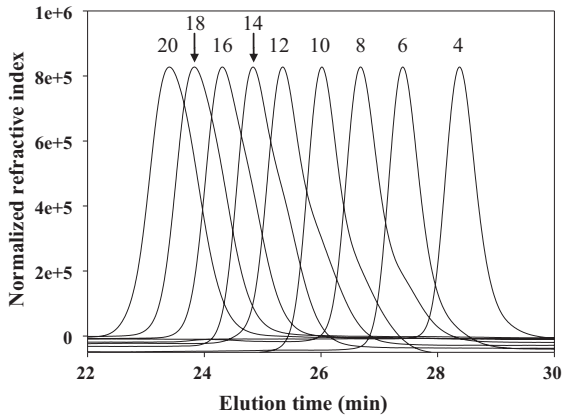

Fig. 2. SEC analysis of the heparin standards. SEC separation was performed on a TSK-GEL 3000 mounted in series with two TSK-GEL 2000 columns at a flow rate of 1 mL/min using 0.1 M ammonium acetate as eluant. The degree of polymerization (DP) is indicated on each curve.

Ultrasonic waves are known to cause ruptures in the liquid phase in the form of small bubbles (cavities) filled with vapor and some cavities violently collapse, inducing high pressure gradients and high local velocities of the liquid layers in their vicinity. This in turn may cause shear forces that have no significant influence on small molecules but are capable of breaking the polymer chains, provided the chains are longer than a certain limiting value. In addition to cavitation, solvent molecules may dissociate to form radicals and diffuse out of the cavity to the surrounding liquid and attack the solute (Czechowska-Biskup et al., 2005). In our case, the combination of hydrogen peroxide with ultrasonic waves is likely to enhance the formation of free radicals that attack the saccharidic chains. Beside these two mechanisms, polymers in solution can undergo pyrolysis in the interfacial region between the cavities and the surrounding liquid, where temperatures increase momentarily when the cavities collapse (Kawasaki, Takeda, & Arakawa, 2007). All the same, these mechanisms are not specific, which explains the structural heterogeneity of the products.

The chromatographic profile of LMWH obtained after ultrasonic-assisted radical depolymerization was similar to those obtained with commercial LMWH. The proportion of oligosaccharides that contained less than 5 saccharide units was low; these are considered to be side products that do not have any anticoagulant activity. The advantage of this physicochemical method is thus that it require less purification step than others methods to eliminate either side products or chemicals used for the reaction (Linhardt & Gunay, 1999). Table 1 shows the number-averaged molecular weight (M_n), weight-averaged molecular weight (M_w), polydispersity index (I), the degree of polymerization (DP) and the degree of sulfation obtained using the different depolymerization methods compared to commercial LMWH. After 1 h of reaction, ultrasonic-assisted radical depolymerization resulted in low molecular weight heparin with a number-averaged molecular weight of 4324 Da and a mass-averaged molecular weight of 5985 Da. The resulting polydispersity index was 1.38, which was comparable to the value measured for commercial LMWH. The dispersity of the heparin polymeric fraction is considered to be a fundamental parameter for a better control of pharmaceutical LMWH solution activities and side effect reduction (Ye et al., 2009). Therefore, the low polydispersity of LMWH obtained from ultrasonic-assisted radical depolymerization could be considered as an important advantage. However, this physicochemical method induced some desulfation of the polysaccharide chain. In fact, LMWH produced by ultrasonic-assisted radical depolymerization had a degree of sulfation of about 0.7 sulfate groups for every two saccharide units, corresponding to about 20% desulfation given that the UFH starting material had a relatively low degree of sulfation of 1.13. It is noteworthy that commercial UFH has variable degrees of sulfation depending on the source of heparin and the extraction or purification process (Naggi & Torri, 1988).

In order to investigate the anticoagulant effect of LMWH obtained from ultrasonic-assisted radical depolymerization, the anti-factor Xa and the anti-IIa activities of LMWH products were

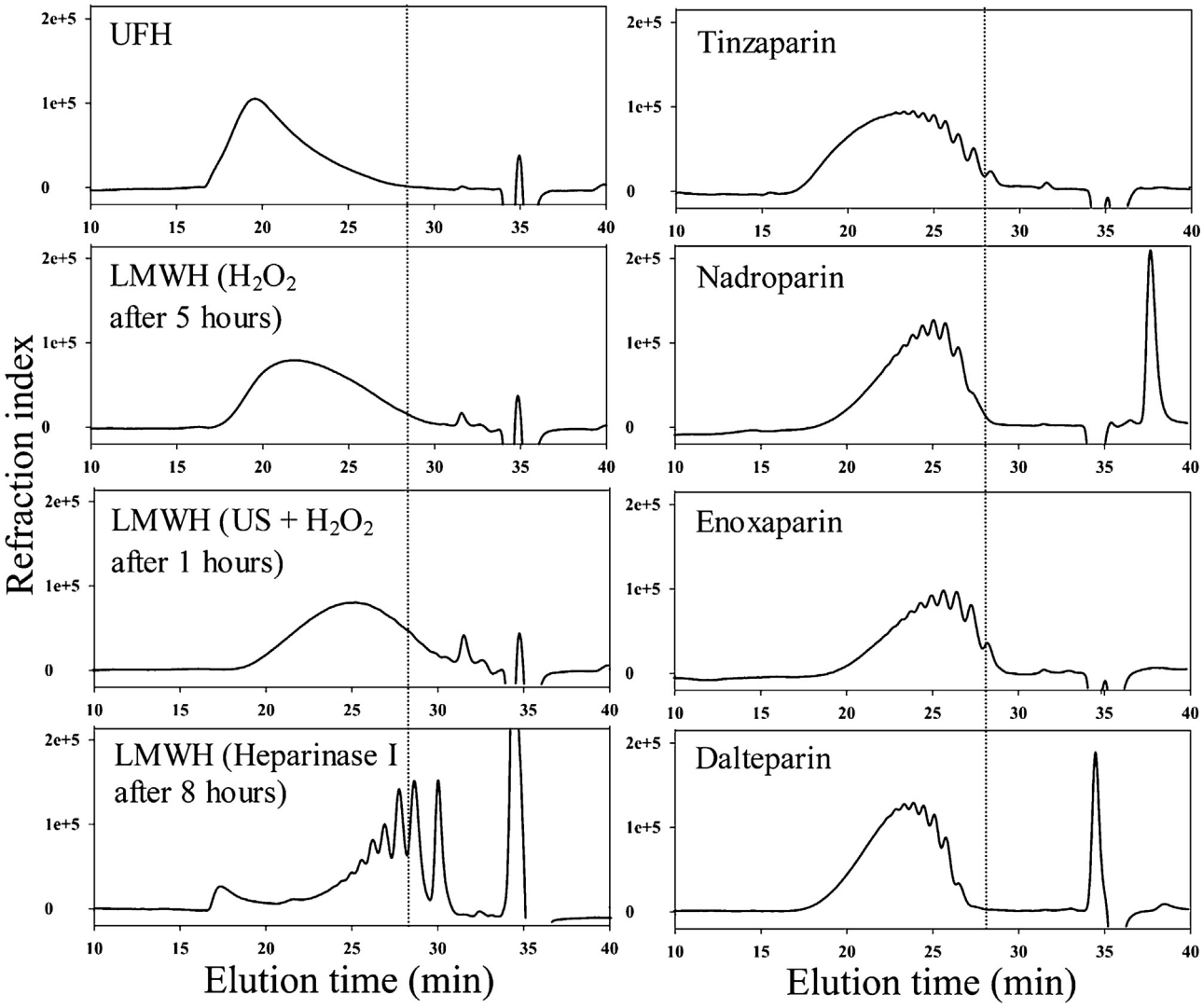

Fig. 3. SEC analysis of crude heparin products before and after depolymerization catalyzed by heparinase I or hydrogen peroxide (H_2O_2) combined with ultrasonic waves (US), and commercial LMWH. Dashed lines indicate the time when pentasaccharide elution is expected. SEC separation was performed on a TSK-GEL 3000 mounted in series with two TSK-GEL 2000 columns at a flow rate of 1 mL/min using 0.1 M ammonium acetate as eluant.

Table 2
Anti-Xa and anti-IIa activities of unfractionated heparin (UFH), commercial LMWH and LMWH produced by depolymerization catalyzed by radical hydrolysis, assisted or not by ultrasonic waves, or by heparinase I.

Depolymerization conditions	Anti-Xa activity (U/mg)	Anti-IIa activity (U/mg)	Anti-Xa/anti-IIa
UFH	167	167	1
LMWH (ultrasound + H_2O_2)	143.62 ± 5.42	77.07 ± 4.4	1.86
LMWH (H_2O_2)	161.65 ± 5.26	111.06 ± 1.85	1.45
Heparinase I	132.26 ± 4.07	96.87 ± 6.87	1.36
Nadroparin (Fraxiparine®)	178.28 ± 9.2	66.90 ± 3.74	2.66
Dalteparin (Fragmine®)	172.07 ± 5.53	87.58 ± 3.39	1.96
Tinzaparin (Innohep®)	181.48 ± 7.31	46.37 ± 3.07	3.91
Enoxaparin (Lovenox®)	183.91 ± 2.13	44.77 ± 3.07	4.10

measured (Table 2). Anti-Xa and anti-IIa activities of heparin products obtained after a one hour of ultrasonic-assisted radical depolymerization were 143.62 U/mg (±5.42) and 77.07 U/mg (±4.4), respectively, with an anti-Xa/anti-IIa ratio of 1.86. This ratio was analogous to the ratio measured for the commercial LMWH dalteparin (Fragmine®). We also noticed that 87% of the anti-Xa activity was retained from the UFH starting material (167 U/mg) under these physicochemical depolymerization conditions. This result suggests that this physicochemical method largely preserves the pentasaccharide ATIII binding sequence. However, the anti-Xa activity of commercial LMWH was higher than that of the UFH starting material and consequently higher than the activity of the ultrasonic-assisted radical depolymerization products.

4. Conclusion

In this work, we highlighted a physicochemical method for the production of LWMH. This method, which combines the use of ultrasonic waves with the hydrogen peroxide-catalyzed radical

depolymerization of heparin, gave rise to LMWH products with molecular weight properties that are similar to those of commercial LMWH. The anti-Xa and anti-IIa activities of LMWH produced by this method were also similar to those of some commercial LMWH. Moreover, these products were obtained after only a one hour reaction. On the others hand, the proportion of oligosaccharides that contained less than 5 saccharide units was low. This physicochemical depolymerization process relies on mild reaction conditions, requires no harsh or toxic reagents, and provides a product free of chemical artifacts: it thus might be applied for the large scale production of LMWH, but also analogs produced from natural sulfated polysaccharides. Future studies will be conducted to optimize the reaction conditions for the production of these compounds, but also to assess their *in vivo* biological activities as well as their chemical structure.

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