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Biosimilar Evaluation and Structural Characterization, A Comparison Study for Enoxaparin

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ABSTRACT

Generic forms of chemically- synthesized drugs must exhibit chemical identity and be bioequivalent in healthy human subjects. Biologic products are 100- to 1,000-fold larger than chemically synthesized drugs, with sophisticated three-dimensional structures, and can be mixtures of isoforms rather than pure homogeneous entities. Therefore, the development process for biosimilars is more complex than for a true generic and the demonstration of approvability for biosimilars differs from the standard generics approach as it is based on a comparability exercise rather than on demonstration of bioequivalence. This study examines the case of a Low-molecular-weight heparins (LMWHs): enoxaparin which is among smallest biological molecules. Different chemical tests such as Nuclear Magnetic Resonance (NMR), Size exclusion Chromatography, Specific absorbance, stability tests and biological assay (anti-factor Xa activity and anti-factor II a activity) were used for a comparability exercise focusing on quality and structural aspects of enoxaparin biosimilar product compared to the reference product. All tests and comparative studies showed no significant difference. in fact the data observed suggests comparable results even under accelerated conditions of stability study. This study suggests that there is no significant difference in the profile structure and overall studied quality aspects of the reference product compared to the similar biological medicinal product. however specific analytical methods as well as additional biological and pharmacological tests may be used to address their interchangeability.

Keywords: Biologic, Biosimilar, comparability, Low-molecular-weight heparins, enoxaparin, Size exclusion Chromatography, Nuclear Magnetic Resonance, Specific absorbance, biological assay.

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INTRODUCTION

Low molecular weight heparins (LMWHs),marketed first in Europe from the second half of'80, represented a new therapeutic measure, notonly alternative and more practical, but also oftenmore effective than standard heparin in manyclinical fields[1]. Enoxaparin is obtained by alkaline depolymerization of heparin benzyl ester derived from porcine intestinal mucosa. knowledge of the molecular diversity of heparin is important for understanding the molecular diversity of LMWH. Heparin is a mixture of linear polysaccharides that are variable in length, consisting of disaccharide repeating units composed of glucosamine and uronic acid (either iduronic or glucuronic acid) with the following linkage sequence: [(1 ~4) a-D-glucosaminyl(1~4) p-D-hexuronosyl]n. Thus, the molecular diversity of heparin comes from the polydispersity of chain length and the diversity of disaccharide units and the corresponding distribution of disaccharide unit sequences in the polysaccharide chains[2]. Enoxaparin structure is characterized by a 2-O-sulfo-4-enepyranosuronic acid group at the non-reducing end and a 2-N,6-O-disulfo-D-glucosamine at the reducing end of the chain. About 20% (ranging between 15% and 25%) of the enoxaparin structure contains an 1,6 anhydro derivative on the reducing end of the polysaccharide chain. The drug substance is the sodium salt. The average molecular weight is about 4500 daltons. The molecular weight distribution is:

<2000 daltons ≤20%

2000 to 8000 daltons ≥68%

>8000 daltons ≤18% [3].

In general, the biologic products are 100- to 1,000-fold larger than chemically synthesized products [4].however there is differences between the biologic products size that have to be taken into consideration during evaluation and comparability exercise for biologics as recommended by different regulations, in fact Enoxaparin is considered among smallest biological molecules. The difference between structure complexity of the different biological molecules has been taken into consideration during elaboration of guidelines to demonstrate similarity and to approve similar biological medicinal products of Enoxaparin. There is a considerable difference for enoxaparin approval approach between different regulatory agencies. US FDA evaluation is mainly based on a rigorous scientific structural similarity approach and EMEA evaluation based on a complete biosimilarity study demanding extensive testing to ensure similar quality, safety and efficacy profile. In 2010, the US Food and Drug Administration (FDA) approved a generic low-molecular-weight heparin without clinical safety or efficacy data under the Abbreviated New Drug

Application ANDA(generic) legal pathway. FDA considered it justified to base the approval of this biologically-derived product on the ANDA pathway which is nothing else than approving it with the criteria applied for a common chemical generic drug for the evidence of sameness. FDA has, however, taken into account the nature of Enoxaparin specifically in their review by adapting these criteria by the following five criteria:

1. Equivalence of physiochemical properties
2. Equivalence of heparin source material and mode of depolymerization
3. Equivalence in disaccharide building blocks, fragment mapping, and sequence of oligosaccharide species
4. Equivalence in biological and biochemical assays
5. Equivalence of in vivo pharmacodynamic profile

FDA approach suggests that analytical and scientific advancements may in certain cases allow the elimination of unnecessary *in vivo* testing in animals and humans[4, 5, 6].

EMA guidelines on similar biological medicinal products containing LMWHs comprise a completely different approach including demonstration of the similar nature of two biological products in terms of quality, safety and efficacy. For quality issue, specific quality aspects and tests for LMWH are not described but refer to the general comparability guidelines "guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: Quality issues"[7]. The other criteria in term of safety and efficacy are well described in a specific guideline "Guideline on non-clinical and clinical development of similar biological medicinal products containing LMWH"[8]. The non-clinical section of the guideline addresses pharmaco-toxicological requirements with in vitro studies including at least comparative evaluations of anti-FXa and anti-FIIa, *in vivo* studies with comparative pharmacodynamic activity, toxicological studies. The clinical section addresses the requirements for pharmacokinetic /pharmacodynamic, efficacy and safety clinical studies as well as pharmacovigilance aspects. EMA guideline on safety and efficacy is changing by the issue of a new draft guideline, with less strict requirements on the necessity of toxicity and clinical studies as taking into consideration physico chemical and biological characterization of enoxaparin. In fact the draft guideline describes in the non clinical section possibility of waiver of the *in vivo* studies as well as toxicological studies. Also in the clinical section strict conditions for waiver of clinical efficacy studies are discussed[9]. This study aim to compare biosimilar enoxaprin product with reference product available in Europe were used for this study two biosimilar enoxaparin products that were compared to the reference product. Similarity and differences between the different biosimilar

enoxaprin drug products and reference product were studied, with a focus on structure and quality aspects, using comparative *in vitro* physicochemical tests and biological tests to evaluate relationship between tests and structure and their impact on drug product therapeutic effect.

MATERIALS AND METHOD

Samples used

Enoxaparin reference drug product at 4000 UI anti-Xa/0,4 ml: Lovenox manufactured by les laboratoires Sanofi-Aventis(France). Enoxaparin similar biological medicinal drug products at 4000 UI anti-Xa/0,4 ml : ENOXA 1 and ENOXA 2 manufactured by les Laboratoires Médis (Tunisia).

Specific absorbance: Uronic acid determination of Enoxaparin

The uronic acid content "Δ 4,5 uronate" of each of Enoxaparin drug product samples was compared by the specific absorbance method using USP monograph « Enoxaparin sodium» that was adequately adapted to the drug product[10]. A standard curve of absorbance at 231 nm of Enoxaparin for each sample and for enoxaparin standard solution was prepared by reconstitution in HClat 0.5mg/ml. A comparison has been performed statistically on 6 samples of each product results are expressed as average and standard deviation.

Identification tests; performed as described in USP monograph « Enoxaparin sodium injection »[11]. Identification A: A specific structural identification reaction was performedwith protamine sulfate solution

Identification B: using specific absorbance test that have been performed as described above

Identification C: a sodium identification test [12].

Determination of the pH of each test solution: test performed using pH meter.

Determination of the density of each test solution: test performed using densimeter.

Size exclusion Chromatography

Molecular weight distribution and average molecular weight was evaluated and compared on each Enoxaparin drug product samples by chromatography exclusion method using European pharmacopoeia monograph,[13] on drug substance adequately transformed to a method on the drug product. Test solution of each product and standard solution were prepared at 10 mg/ml, mobile phase was a 28.4 g/l solution of anhydrous sodium sulphate R adjusted to pH 5.0 using diluted sulphuric acid R, the Column was as follow: l=0.30m ; Ø =7.5mm with a silica gel (5 µm)stationary phase: number of theoretical plates: minimum of 20 000 per meter, and detection with differential refractometer. Each test solution (25µL, 10 mg/ml) were injected , Flow rate of

0.5 ml/min is used. This comparison has been performed statistically on 6 samples of each drug product.

C 13Nuclear Magnetic Resonance (NMR) spectrum

NMR spectrum was evaluated comparatively on each Enoxaparin drug product samples using a European pharmacopoeia method on drug substance that was transformed to a method on drug product: Reference solution and test solution were prepared at 80 mg/ml adding a solution of deuterium oxide. The reference solution and the different test solutions were transferred to NMR tubes of 5-mm diameter. Using a pulsed (Fourier transform) NMR spectrometer operating at not less than 75 MHz for 13C, the 13C NMR spectra of the reference solution and the Test solutions at 40 degree were recorded[13].

Biochemical assay for anticoagulation activity of Enoxaparin

Anticoagulation activity of Enoxaparin was performed by *in vitro* determination of factor Xa inhibition and factor IIa inhibition the method is described in European pharmacopoeia for drug substance and was adequately adapted to the drug product[13].

ANTI-FACTOR Xa ACTIVITY

Preparation of solutions

A buffer solution 1 at pH 7.4 using of tris(hydroxymethyl)aminomethane, sodium chloride and hydrochloric acid and a buffer solution 2 at pH 8.4 using tris(hydroxymethyl)aminomethane , sodium chloride and edetate sodium and hydrochloric acid were prepared.

Antithrombin solution III R1 and Xa bovin R factor solution were prepared by simple dissolution in water. chromogenic Substrate R1 solution was prepared by dissolution in water then dilution in buffer solution 2. reference solutions at 1 UI anti Xa/ml of biological product reference supplied by European pharmacopeia was prepared by dilution in buffer solution 1

solution to be tested containing reference product or similar biological medicinal product were prepared by dilution at 1UI/ml using buffer solution 1

- Four tubes of the dilution of the test solution were prepared (using buffer 1 solution) in duplicate T1, T2, T3, T4 (0.05 UI/ml, 0.075 UI/ml, 0.1 UI/ml, 0.125UI/ml) and four tubes of the dilution of the reference solution were prepared in duplicate S1, S2, S3, S4(0.05 UI/ml, 0.075 UI/ml, 0.1 UI/ml, 0.125UI/ml), then two tubes filled with buffer solution 1were prepared as a blank (B).

To each tube was added 50 µl of antithrombin III solution R1 and 50 µl of the appropriate dilution of the test solution, or the reference solution, then were mixed, avoiding the formation of bubbles.

The order of treatment was B,S1, S2, S3, S4, T1, T2, T3, T4, T1, T2, T3, T4, S1, S2, S3, S4 and B

After equilibration at 37 °C (water-bath or heating block) for one minute, 100 µl of bovine factor Xa solution R was added in each tube and left incubating for exactly one minute before adding 250 µl of chromogenic substrate R1. After exactly four minutes, the reaction was stopped by adding 375 µl of acetic acid Rand the mixtures were transferred to semi-micro bowl, then, the absorbance was measured at 405 nm using a suitable reading device.

The regression of the absorbance on log concentrations of the test solutions and of reference solution of low-molecular-weight heparins was calculated and potency of the test solution in International Units of anti-factor Xa activity per milliliter was calculated using the usual statistical methods for parallel-line assay.

This comparison was completed by additional tests that have been performed statistically on 4 samples of each product of under same conditions.

ANTI-FACTORIIa ACTIVITY:

Preparation of solutions:

Antithrombin solution III R2 and bovine thrombin R solution were prepared by simple dissolution in water.

Chromogenic substrate R2 solution was prepared by dissolution in water then dilution in buffer solution 2.

Reference solutions at 1 UI anti IIa/ml of biological product reference supplied by European pharmacopeia was prepared by dilution in buffer solution 1

Solution to be tested containing reference product or similar biological medicinal product were prepared by dilution at 1UI/ml using buffer solution 1

- Four tubes of the dilution of the test solution were prepared (using buffer 1 solution) in duplicate T1, T2, T3, T4 (0.02 UI/ml, 0.035 UI/ml, 0.05 UI/ml, 0.065UI/ml) and four tubes of the dilution of the reference solution were prepared in duplicate S1, S2, S3, S4 (0.02 UI/ml, 0.035 UI/ml, 0.05 UI/ml, 0.065UI/ml), then two tubes filled with buffer solution 1were prepared as a blank (B).

Each tube was filled with 50 µl of antithrombin III solution R2 and 50 µl of the appropriate dilution of the test substance or the reference preparation, then were mixed, avoiding the formation of bubbles. The order of treatment was S1, S2, S3, S4, T1, T2, T3, T4, T1, T2, T3, T4, S1, S2, S3, S4, After equilibration at 37 °C (water-bath or heating block) for one min, 100 µl of bovine thrombin R solution was added in each tube and were left incubating for exactly one minute before adding 250 µl of chromogenic substrate R2. After exactly four minutes, the reaction was

stopped by adding 375 µl of acetic acid R. and the mixtures were transferred to semi-micro bowl, then, the absorbance was measured at 405 nm using a suitable reading device.

The regression of the absorbance on log concentrations of the test solutions and of the reference solution of low-molecular-weight heparins was calculated and potency of the test solution in International Units of anti-factor IIa activity per milliliter was calculated using the usual statistical methods for parallel-line assays. This comparison was completed by additional tests that have been performed statistically on 4 samples of each product.

Statistical analysis:

Statistical analysis of the data was carried out using usual statistical methods for parallel-line assay, then additional Statistical analysis for comparison using Cochran test

Comparative stability testing

Stability profile was compared between each Enoxaparin drug product using International Conference of Harmonization guideline [14]. stability under accelerated conditions was performed with a temperature of 40 ±2 °C and relative humidity of 75 ± 5% for a period of six months. The following tests were performed : Aspect, anti- Xa activity, anti IIa activity, anti Xa/anti IIa ratio, pH for the testing points :zero month, three months and six months.

RESULTS AND DISCUSSION

Specific absorbance: Uronic acid determination of Enoxaparin

The results were complying and showed sameness (Table I); a Statistical analysis using Cochran test has been added and showed that for each sample (test product) results are acceptable as <0.7 (cocheran table). These results showed equivalency of the Specific absorbance for the three samples Enoxa 1/ Enoxa 2 and Lovenox, Equivalency of ultraviolet (UV) specific absorbance demonstrate the presence of unique functional groups such as the Δ 4,5 uronate structure known to be present in Enoxaparin. In fact unlike heparin, LMWHs may have unique chemical modifications (or “fingerprints”) at the non reducing and reducing ends (or terminal ends)of the oligosaccharide chains, which provide an additional, third dimension of molecular diversity not present in Heparin. These unique chemical fingerprints at the terminal ends of the chains are a result of the mode of depolymerization used to manufacture the LMWH. For instance, Enoxparin sodium is derived by estirification of Heparin derived from porcine intestinal mucusa to the corresponding heparin benzyl ester. the intermediate Heparin benzyl ester undergoes alkaline ß-elimination cleavage. This results in selective cleavage between the uronic and glucosamine residues and fragmentation of Heparin polysaccharides into smaller oligosaccharide fragments,

with the majority of the components having a 4,5 delta (Δ 4,5)- uronate structure at the non reducing end of the chain[15,16]

Table 1: Results of the specific absorbance comparative test for different samples on each drug product

Product	Specific absorbance As	Average	Standard deviation
Enoxa 1	2,0223	2.0180	0.0083
	2.0247		
	2.0033		
	2.0 28		
	2.0223		
	2.0223		
Enoxa 2	2.0266	1.9870	0.0252
	1.9624		
	1.9791		
	1.9600		
	1.9933		
	2.0005		
Lovenox	1.9148	1.8356	0.1059
	1.6841		
	1.9172		
	1.7198		
	1.8649		
	1.9125		

Identification tests

All identification tests were complying for each drug product: Enoxa 1; Enoxa 2 and Lovenox. dentification A: Precipitation with protamine sulphate was observed with all samples; also identification B was positive for all samples at Absorbance 230 – 233 nm and identification C has shown positive reaction for all samples

Determination of the pH of each test solution

For Enoxa 1 pH is 6.25; for Enoxa 2 pH is 6,2; for Lovenox pH is 6.53

Results of pH for all samples were complying to the specification described in USP monograph: pH must be between 5.5 and 7.5.

Determination of the density of each test solution

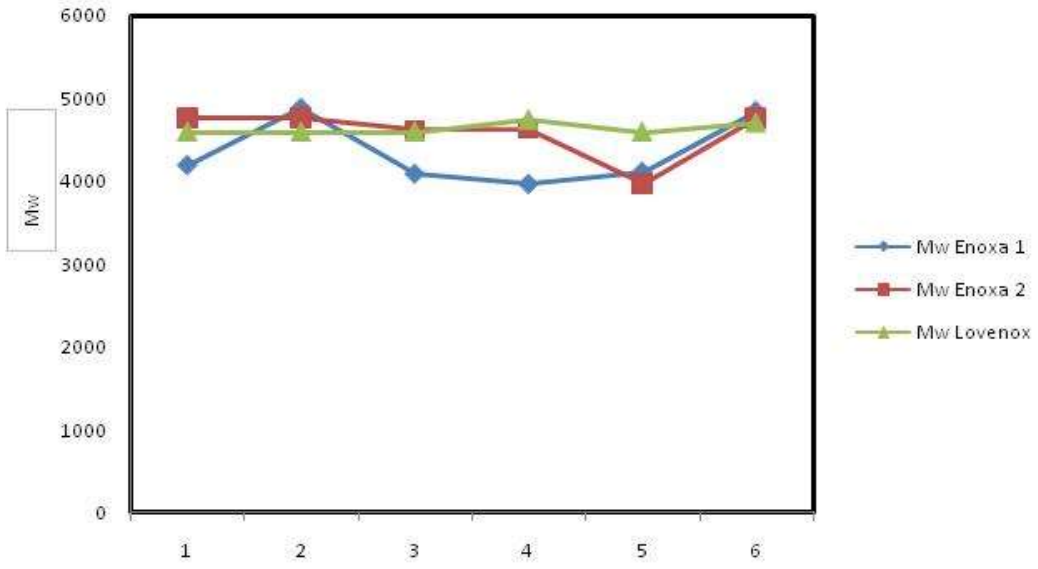
For Enoxa 1 density is 1.052; for Enoxa 2 density is 1.051; for Lovenox density is 1.051

Results of density for all samples were complying to the specification: Density must be between 1.04 and 1.08.

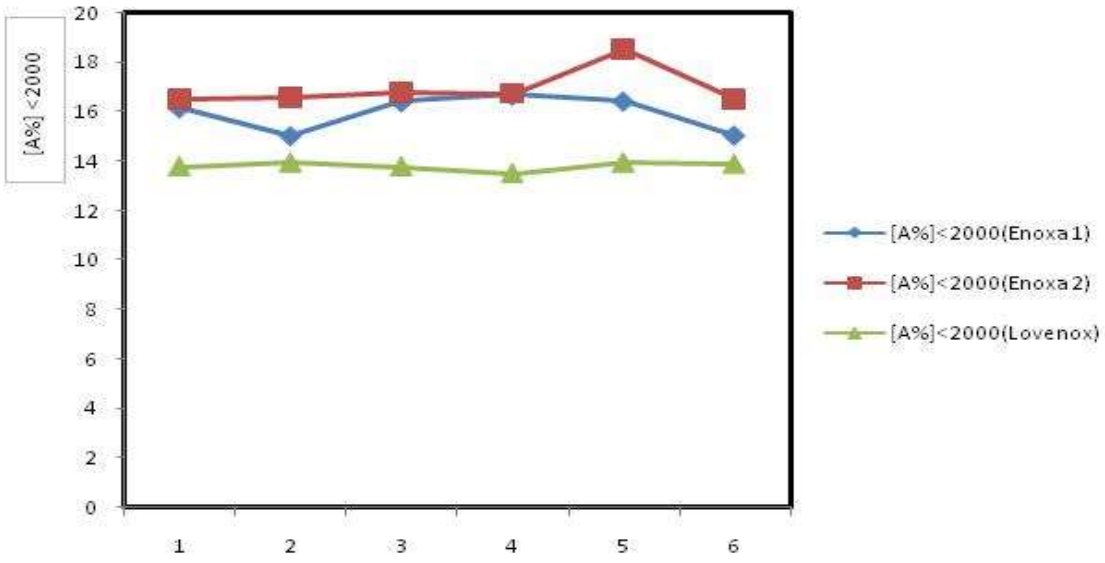
Size exclusion Chromatography

The results of different samples showed sameness (figure 1). Statistical analysis using Cochran test show that there is no inter-groupe variation between the two similar biological medicinal products and reference product; The molecular weight distribution determination provides information on the extent and pattern of depolymerization of heparin , relative abundance of oligosaccharides of different molecular weights that are included in enoxaparin and thus equivalency of the oligosaccharide chain lengths, including their distribution and proportion[15,17].

(A)



(B)



(C)

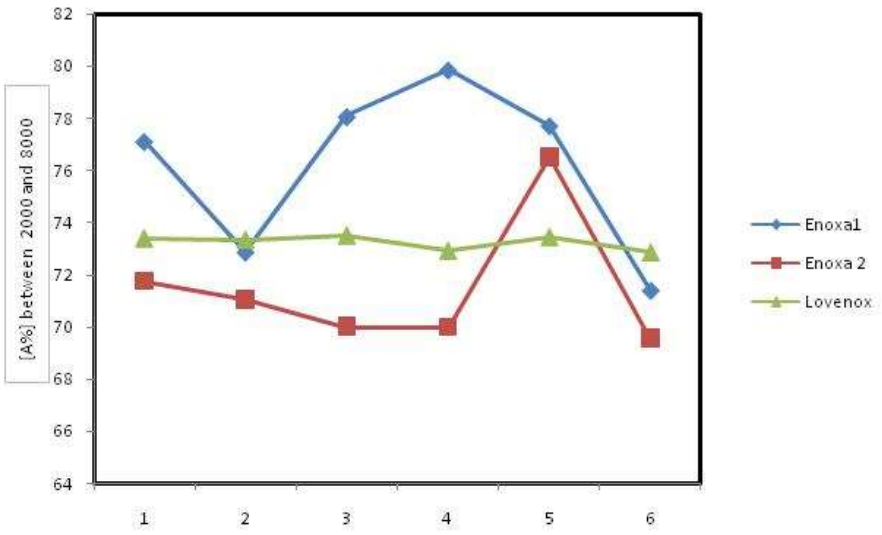
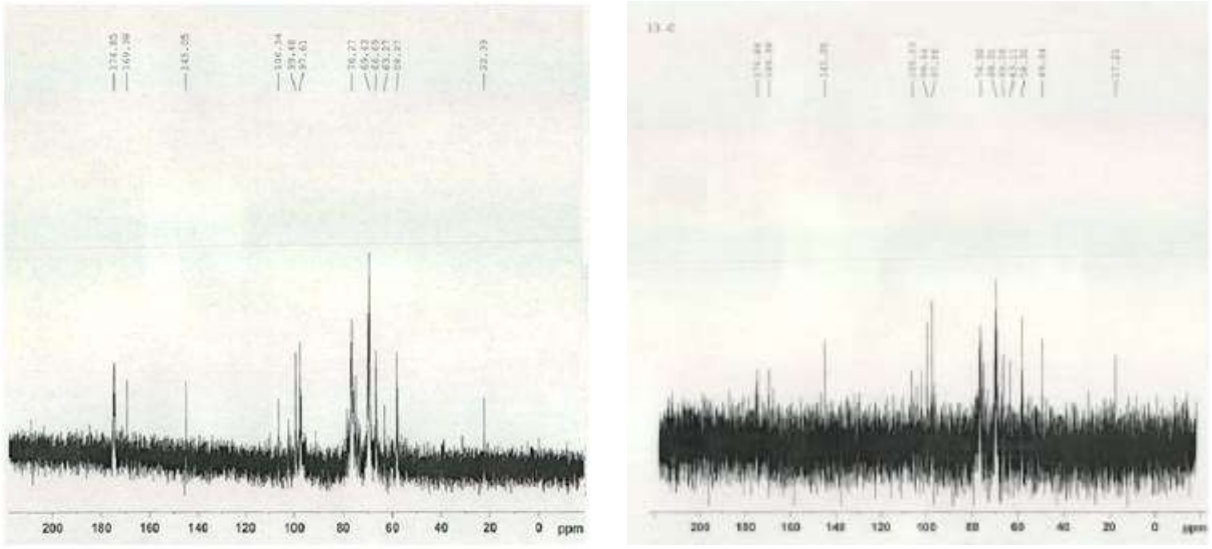


Figure 1 : (A) molecular weight distribution for different enoxaparin drug products on 6 samples of each**(B)** % in weight of the chains having molecular weight<2000 Da for different enoxaparin drug products on 6 samples of each**(C)** sum of % in weight of the chains having molecular weight between between 2000 and 8000 Da for different enoxaparin drug products on 6 samples of each.

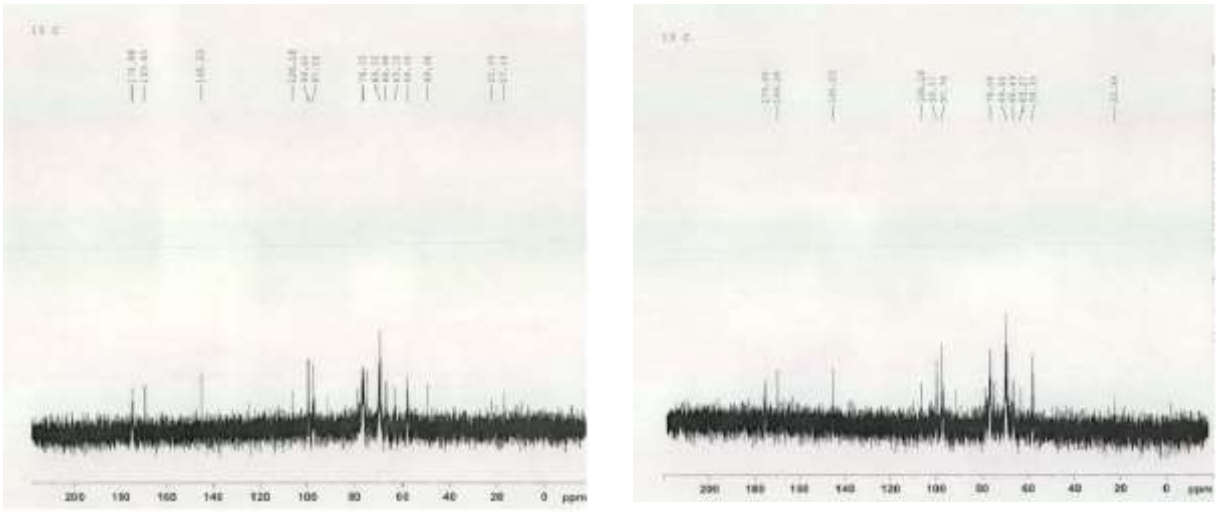
C 13Nuclear Magnetic Resonance (NMR) spectrum

Results shown in (figure 2) showed comparable signals that suggests comparable structure of enoxaparin similar drug products and enoxaparin reference product, also it shows no contamination by contaminant such as over sulfated chondroitin sulfate (OSCS), that has in 2007–2008 spawned a global crisis of contaminated heparins and LMWHs[17,18].

(a) (b)



(c) (d)



(e) (f)

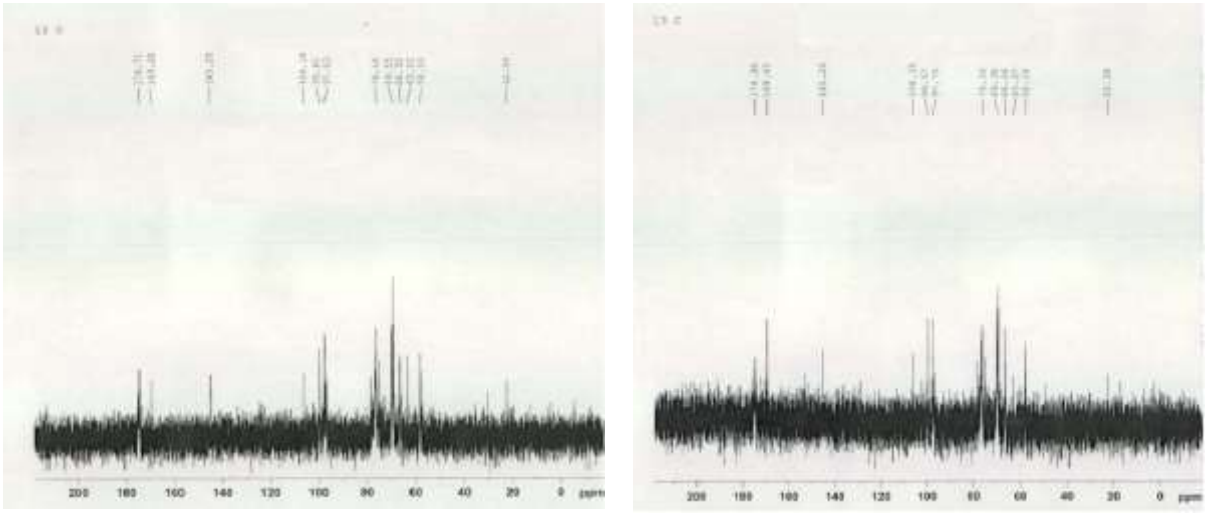


Figure 2: Comparative NMR spectra of the different Enoxaparin drug products, heparine and enoxaparine reference substances:(a) Heparin reference; (b) Product 1 (c) Product2, (d) Lovenox batch 1, (e) Lovenox batch 2, (f) Enoxaparin reference

Biochemical assay for anticoagulation activity of Enoxaparin

Data obtained for this biochemical assay are shown in table II; Results suggest comparable data between the two Enoxaparin similar biological medicinal drug products and enoxaparin reference drug product. The molecular basis for factor Xa inhibition (anti-Xa) is mediated by an AT-III binding pentasaccharide sequence motif that is present in heparin and LMWHs. This particular protein binding motif, which is present in approximately 30 percent of heparin polysaccharide chains and 15 to 25 percent of LMWH oligosaccharide chains, has a high affinity for AT-III, an endogenous serpin inhibitor. Binding of this pentasaccharide sequence motif to AT-III results in an

activated AT-III-polysaccharide complex that inhibits the proteolytic activity of factor Xa in the coagulation cascade. Also the molecular basis for factor IIa inhibition (anti-IIa) is mediated by this particular AT-III binding pentasaccharide sequence[19].

Table 2: Enoxaparin biochemical comparative assay: anti IIa, anti Xacomparison

Product	sample	anti Xa Between 9000 and 11000 IU /ml	conclusion	anti IIa Between 2000 and 3500 IU/ml	conclusion	anti Xa/anti IIa Between 3.3 and 5.3	conclusion
ENOXA 1	1	10045.85	comply	2653.01	comply	3.786585803	comply
	2	9841.52	comply	2863.76	comply	3.436572897	comply
	3	9804.59	comply	2686.11	comply	3.650107404	comply
	4	9989.98	comply	2717.01	comply	3.676828573	comply
ENOXA 2	1	9928.3	comply	2492.23	comply	3.983701344	comply
	2	10115.11	comply	2586.08	comply	3.911367784	comply
	3	10038.43	comply	2363.64	comply	4.247021543	comply
	4	10118.47	comply	2304.91	comply	4.389963166	comply
LOVENOX	1	9746.95	comply	2402.92	comply	4.056294009	comply
	2	10036.14	comply	2338.49	comply	4.29171816	comply
	3	9868.72	Comply	2352.36	comply	4.195242225	comply
	4	9514.09	Comply	2659.23	comply	3.577761232	comply

Comparative stability testing

A comparable stability profile was shown (Table 3) under accelerated conditions and helped to conclude about similar profile under high humidity and temperature, concluding that both products should be stored under 25°C[14,20].

Table 3: Enoxaparin drug products comparative stability study

Tests	Aspect		anti- Xa		anti IIa		anti Xa/anti IIa ratio		pH	
Limits	Limpid Liquid colorless to yellowish JB4		9000 to11000 UI/ml		2000 to 3500 IU/ml		3.3 to 5.3		5.5 to 7.5	
Products	Enoxa 1	Lovenox	Enoxa 1	Lovenox	Enoxa 1	Lovenox	Enoxa 1	Lovenox	Enoxa 1	Lovenox
0 month	Comply	Comply	10241	9972	2695	2716	3.8	3.7	6.9	6.5
3 months	Comply	Comply	9942	9773	2627	2721	3.8	3.6	6.7	6.4
6 months	Not comply	Not comply	9273	9778	2503	2692	3.7	3.6	6.3	6.3

CONCLUSION

The tests performed during this comparative study such as : ultraviolet (UV) specific absorbance molecular weight distribution, NMR spectrum and overall chemical tests , Biochemical assay for anticoagulation activity anti Xa and anti IIa test, suggested comparable results. These data showed at this stage of comparability exercise that there is no significant difference in the profile structure

and overall quality aspects of the reference product compared to the similar biological medicinal products. However additional specific analytical methods as well as additional biological and pharmacological tests may be used to address their interchangeability. It is also important to understand limits and complexity of interpretation of such comparability results for marketing authorization approval due to the nature of biological products and international regulation different approach for their approval with lack of harmonized procedures.

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