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OBTAINING THE SUBSTANCE ENOXAPARIN SODIUM EQUIVALENT TO THE ORIGINAL Clexane® AND Lovenox®. SELECTION OF TECHNOLOGICAL PARAMETERS OF THE KEY STAGE OF THE SYNTHESIS

Yuliia Bovsunovska, Vitalii Rudiuk, Volodymyr Mishchenko, Victoriya Georgiyants

The aim: *to carry out the key stage of synthesis to obtain a substance equivalent to the original drugs Clexane® and Lovenox® by determining the technological parameters of the synthesis that are critical from the point of view of the formation of the molecule and studying the correlation between the structural characteristics of Enoxaparin samples and the experimental conditions of the technological process.*

Materials and methods: *samples of the Enoxaparin sodium substance were synthesized according to the method described in the patent, as well as with a variation of the selected critical technological parameters. The obtained samples of Enoxaparin sodium were analyzed according to pharmacopoeial requirements, as well as by non-pharmacopoeial methods, such as two-dimensional NMR spectroscopy and size exclusion chromatography for detailed structural characterization of the molecule.*

Results: *determination and variation of technological parameters critical for the formation of the molecule, such as temperature, the amount of alkali for the depolymerization reaction, and the reaction time of the reaction mass, were determined and varied. Enoxaparin sodium samples were developed according to the selected parameters and a detailed analysis of the structure of the obtained samples was carried out, followed by a comparison with the original Clexane® and Lovenox®. It was established that with an increase in the temperature of the reaction mass, the amount of alkali and the holding time individually and in combination, the degree of depolymerization increases, which makes the composition of the molecule unbalanced in comparison with the original drugs Clexane® and Lovenox®.*

Conclusions: *As a result of the experiments, the technological parameters of the synthesis of a sample of Enoxaparin sodium were evaluated and determined, allowing to obtain a substance comparable to the originator in terms of chemical structure (alkali/ heparin benzyl ester ratio 0.06; temperature – 57 °C, reaction mixture holding time – 1.5 hours)*

Keywords: *Enoxaparin, Low molecular weight heparin, technological parameters, compositional analysis, HSQC, size-exclusion chromatography, reducing, non-reducing ends*

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1. Introduction

Enoxaparin sodium is a sulfated glycosaminoglycan, a semi-synthetic low-molecular weight (3500–5000 Da) analogue of heparin, which is used for chemoprophylaxis and treatment of deep vein thrombosis and venous thromboembolism [1] and is included in the WHO Model List of Essential Medicines.

Enoxaparin sodium was developed in 1981 and is the first drug from the group of low-molecular-weight heparins, approved and authorized for use in France in 1987. Today, the original and, at the same time, the main manufacturer of Enoxaparin is Sanofi Aventis, which produces the product under the trade names Clexane® and Lovenox®. In addition, the substance Enoxaparin sodium is produced by many pharmaceutical companies in the world. The increase in the use of Enoxaparin sodium and even its short-term shortage on the global pharmaceutical market in the last few years was facilitated by its effectiveness in the treatment of patients with COVID-19 [2, 3].

As a starting material for the synthesis of Enoxaparin, heparin is used – a heterogeneous mixture of highly sulfated oligosaccharides belonging to the glycosaminoglycan family [4]. It consists of repeating disaccharide blocks, which include D-glucosamine and D-glucuronic and/or L-iduronic acids connected by glycosidic bonds [5]. The structure of the heparin molecule consists of alternating saccharide units that form chains of different lengths and molecular weights. The heterogeneity is explained by the specificity of the biosynthesis of the substance and various types of microheterogeneity, which is the result of incomplete enzymatic reactions [6]. And that is why heparin was replaced by low molecular weight heparins (LMWH), due to their improved pharmacokinetic properties, safety and efficacy [7]. LMWH is usually obtained by chemical or enzymatic depolymerization, which gives complex and polydisperse mixtures of

oligosaccharides, which can be structurally modified at non-reducing and/or reducing ends because of the cleavage process [8, 9]. The obtained mixture of polydisperse oligosaccharides has a greater degree of complexity of the molecular structure due to side reactions that change the endogenous disaccharide skeleton during depolymerization [10, 11].

In 2008, a series of reports were recorded in the United States of at least 100 fatal cases, to a lesser extent, 800 serious allergic reactions associated with the use of heparin sodium manufactured by Baxter Healthcare Corporation, whose production facilities were located in China [12].

In the context of the so-called heparin crisis, there was a worldwide harmonization of quality requirements for heparin and its low-molecular-weight analogues, because, despite at least 10 different tests of the pharmacopoeial monograph, oversulfated chondroitin sulfate cannot be detected by these methods, since its structure is very similar to the structure heparin, and pharmacopoeial methods do not involve studying the substance at the molecular level [13]. This created a situation in which it was necessary to introduce additional research methods as two-dimensional NMR, enzymatic cleavage of the molecule followed by HPLC analysis and mass spectrometry [14–16], which would provide a more extensive study of such complex molecules as heparin. It also influenced further approaches to the analysis of all heparin analogues, in particular Enoxaparin.

Given the heterogeneity of the molecule and the difficulties in ensuring the quality of LMWH, the European Medicines Agency (EMA) issued a directive for the development of biosimilar LMWH [17]. Due to this Directive the necessary requirement is conducting clinical studies to prove the clinical effectiveness and similarity of the generic with the original drug. Instead, the US Food and Drug Administration (FDA) identifies other criteria for describing equivalence, which it considers more sensitive for identifying differences between branded and new Enoxaparin [18]. Among the 5 main criteria defined by the FDA for recognizing a generic drug Enoxaparin as equivalent to the original, the first three relate to the study of the structure and synthesis technology. In particular, this is the equivalence of physical and chemical properties, the method of depolymerization, the equivalence of disaccharide building blocks. But the FDA notes the criteria of pharmacological equivalence as equally important [19].

Enoxaparin sodium is produced by alkaline hydrolysis of the previously obtained benzyl ester of heparin (Fig. 1) and is unique due to the presence of bicyclic 1,6-anhydro derivatives formed as a result of β-elimination on the reduced fragments of all oligosaccharides containing 6-O-sulfo groups on the glucosamine residue [20]. On average, the Enoxaparin molecule contains 15–25 % of 1,6-anhydrostructures at the reducing ends [21]. The quantitative content of 1,6-anhydrocycles is very important for the pharmacological properties of Enoxaparin sodium – both anticoagulant and not related to the function of blood coagulation.

The influence of 1,6-anhydro structures on the activity of Enoxaparin was determined in a study [23], which compared Enoxaparin sodium with a regulated content of 1,6-anhydro structures with Enoxaparin sodium, where the content of 1,6-anhydro was significantly lower and significantly higher. In the comparison, it was determined that samples with deviations in the amount of 1,6-anhydro have significant differences in anticoagulant and anti-inflammatory effects. It is this stage from the point of view of the oligosaccharide composition, the formation and number of specific structures – 1,6-anhydro derivatives, the number and position of sulfo groups that has the greatest impact on the biosimilarity of the final product.

In order to achieve the set goal – the synthesis of Enoxaparin sodium, similar to the original Clexane® and Lovenox®, it is necessary to select the suitable conditions for its synthesis. At the same time, the result can be affected by several parameters at once – the amount of alkali, the temperature and the time of holding the reaction mixture. Research on establishing the optimal combination of these parameters allows for compliance with the first three FDA equivalence criteria, which specifically relate to the structure of the Enoxaparin sodium molecule.

This study aimed to carry out the key stage of obtaining Enoxaparin sodium by adjusting the technological process (selection of the optimal combination of synthesis parameters) and studying the correlation between the synthesis parameters and the characteristics of the obtained substance to achieve maximum equivalence to the original product.

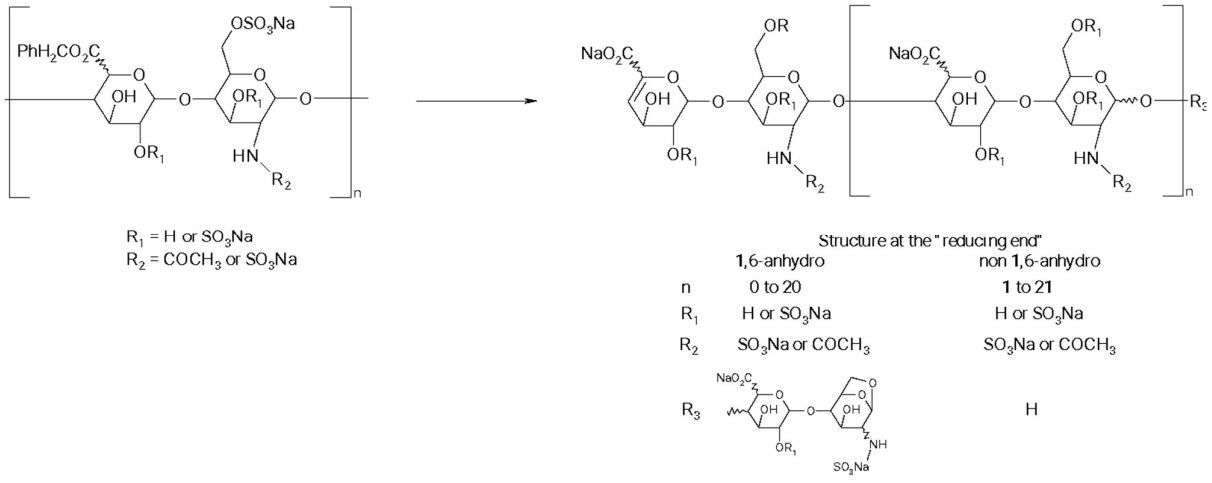


Fig. 1. The key stage of Enoxaparin sodium synthesis [22]

2. Research planning (methodology)

The research was based on the synthesis method described in the patent [22].

According to this method, the depolymerization of heparin benzyl ester (10 g) at the key stage is carried out with sodium hydroxide (0.09 g) at a temperature of 62 °C for 1.5 hours.

The task was to investigate the influence of variations in critical parameters – holding time, the amount of alkali for the depolymerization reaction and the reaction temperature on the quality of Enoxaparin sodium.

The study included the following stages:

1. Determination of "standard" synthesis conditions. At this stage, samples of crude Enoxaparin sodium are obtained according to the methodology given in the patent and according to a changed combination of synthesis parameters. Determine the compliance of the product with pharmacopoeial requirements (after purification).

2. Optimization of "standard" synthesis conditions.

2. 1. Determination of the optimal combination of the amount of alkali and temperature. For the selected variation, investigate the conditions with minor deviations in the parameters: the temperature of the reaction mass ±5 °C, the alkali/ester ratio ±0.01 g.

2. 2. Determination of the optimal holding time. For the best combination of alkali amount/temperature, conduct a study with varying holding time.

At this stage, the patterns of influence of individual parameters on the properties of the obtained products are determined and their optimal combination is selected. The evaluation of samples of crude Enoxaparin sodium after purification is carried out according to pharmacopoeial requirements.

To study the structure of the obtained samples at the second stage and compare them with the originator, additional methods are involved: 2D-NMR (Heteronuclear single quantum coherence spectroscopy) for a comparative study of the compositional composition and size-exclusion chromatography to determine the distribution of oligosaccharide chains.

3. Materials and methods

This study was conducted during 2019–2021.

Clexane from Sanofi-Aventis was obtained from commercial suppliers. All samples were analyzed prior to their expiration dates.

pH Test was carried out on Mettler Toledo Seven compact S220 pH-meter (Switzerland) (Ph. Eur. 2.2.3), Loss on drying test was performed on (Ph. Eur. 2.2.32), Residual solvents test was performed by a method of headspace gas chromatography on Agilent GC 7890B (USA), column DB – 624 sized 60 m×0.32 mm, with a layer thickness of 1.8 µm (Ph. Eur. 2.2.28, 2.2.46), Identification (Molecular Weight Distribution and Weight-Average Molecular Weight was performed on Shimadzu chromatograph (Japan), column PX_TSKgel G2000SW (300 mm*7.8 mm*5 mkm) with detector module Viscotec 305, Malvern Instruments LTD (England) (Ph. Eur. 2.2.30). Analysis by non-pharmacopoeial methods of 2D-NMR (Heteronuclear single quantum coherence spectroscopy) and size-exclusion chromatography was carried out by specialists of the Ronzoni Institute (Italy).

Synthesis of crude Enoxaparin sodium [22] (stage of depolymerization of heparin benzyl ester).

The heparin benzyl ester (10 g) as a sodium salt was dissolved in water (250 ml). To this solution, heated to 62 °C, sodium hydroxide (0.9 g) was added. The temperature was maintained for 1 hour, 30 minutes, at 62 °C. The reaction mixture was then cooled to about 20 °C and neutralized by adding dilute hydrochloric acid. The concentration of the reaction medium was then adjusted to 10 % with respect to sodium chloride. The product was finally precipitated in methanol (750 ml), filtered off and dried.

All samples were obtained according to the given method. The selected parameters were subject to variation in different combinations – the amount of alkali, temperature and holding time. The investigated parameters and their combinations for individual samples of crude Enoxaparin sodium are specified in the text or given in tables.

The obtained samples of depolymerized benzyl ester (crude Enoxparin sodium) were analyzed according to the internal specification developed on the basis of the pharmacopoeial monograph according to the following indicators: Appearance, pH, Loss on drying and residual solvents as indicators of the quality of drying the intermediate product from solvents, Identification (Molecular Weight Distribution and Weight -Average Molecular Weight) – for traceability of changes in molecular weight during the next depolymerization reaction. For detailed structural characterization of samples of crude Enoxaparin sodium obtained under different conditions, analysis was performed by 2D-NMR (Heteronuclear single quantum coherence spectroscopy) and size-exclusion chromatography methods. The results of the analysis were compared with the results of the analysis of the original Clexane, referring to the database formed by the Ronzoni Institute.

4. Research results

4. 1. Definition of "standard synthesis conditions"

To determine the conditions of synthesis, which can potentially affect the "correct" formation of the molecule, a variation of the parameters existing in literary sources was carried out. Based on previous studies, the following variations of critical parameters were selected for consideration:

1. Alkali concentration (alkali/ester mass ratio – 0.09), Temperature – 62 °C, holding time – 1.5 hours – according to patent [22] (**D400**)

2. Alkali concentration (alkali/ester mass ratio – 0.07), Temperature – 62 °C, holding time – 1 hour (**D466**)

The results of experimental studies showed that when reproducing the synthesis parameters described in the patent, the final product after purification did not meet the pharmacopoeial requirements for the "Average relative molecular weight" parameter. This mass turned out to be lower than the minimum permissible level (3800), which indicates a too high degree of depolymerization. Instead, reducing the amount of alkali in sample **D466** leads to a product that, after purification, met the requirements of the EP according to the selected param-

eters. Therefore, these conditions were accepted as "standard" and included in a further experiment on the study of the effect of technological parameter variation on the similarity of the obtained product to the originator.

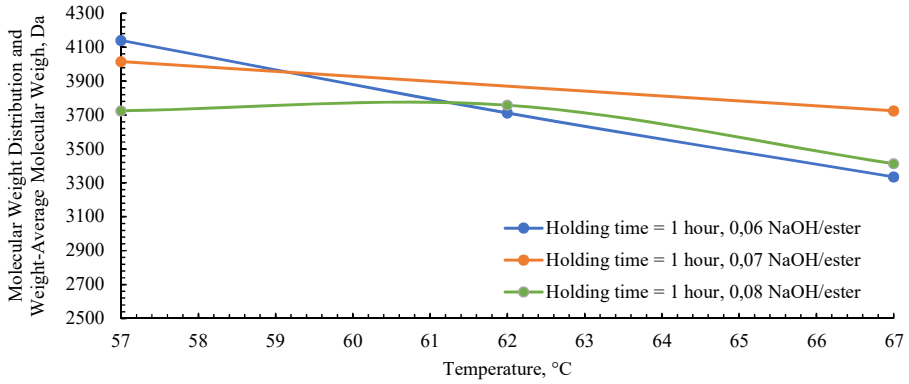
4. 2. Optimization of standard synthesis conditions

4. 2. 1. Variation of alkali concentration and temperature

The research was carried out on 9 samples with the amount of sodium hydroxide 0,6, 0,7 and 0,8 g. For each of these concentrations, the temperature values during exposure were 57 °C, 62 °C, 67 °C. Conventional designation of samples with different variations in the amount of alkali and temperature during synthesis is indicated in Table 1. The holding time for all samples was 1 hour. Evaluation of the received samples according to the laboratory specification, which met the requirements of the Enoxaparin sodium EP monograph (Table 1), showed the non-compliance of some samples with the requirements of the EP according to the "Identification" parameter. The dynamics of changes in the Molecular Weight Distribution and Weight-Average Molecular Weight indicators with increasing temperature for each alkali concentration are shown in Fig. 2.

The composition of the prepared samples was determined by the method of two-dimensional NMR spectroscopy (Table 2).

Obtained profiles of the distribution of fractions in synthesized samples of crude Enoxaparin by size-exclusion chromatography (Fig. 3).



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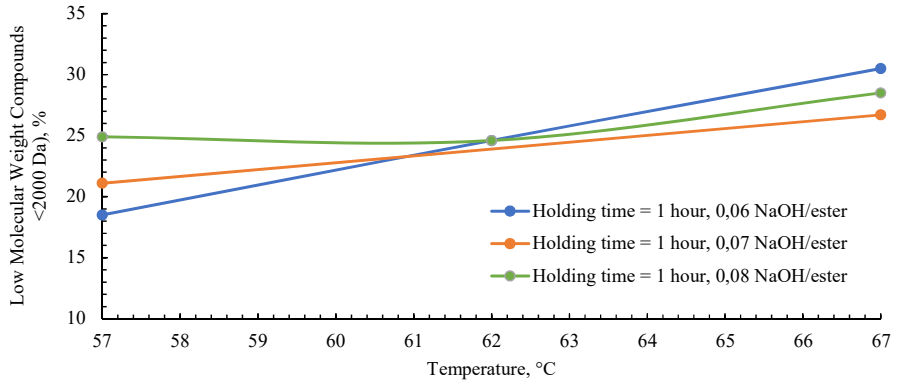

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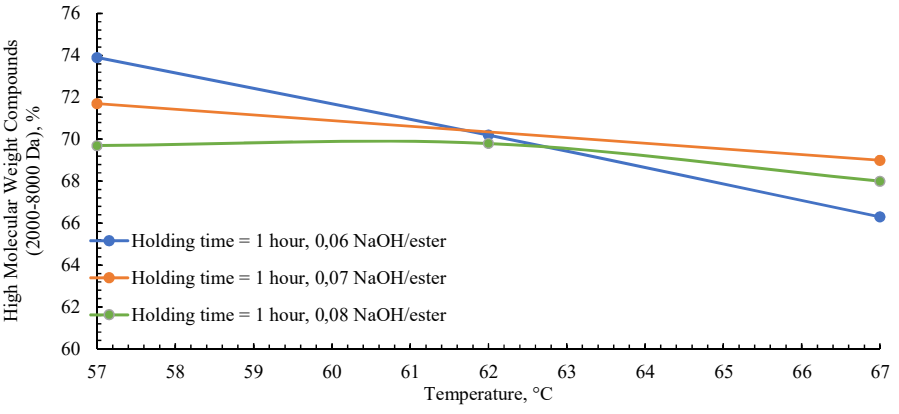
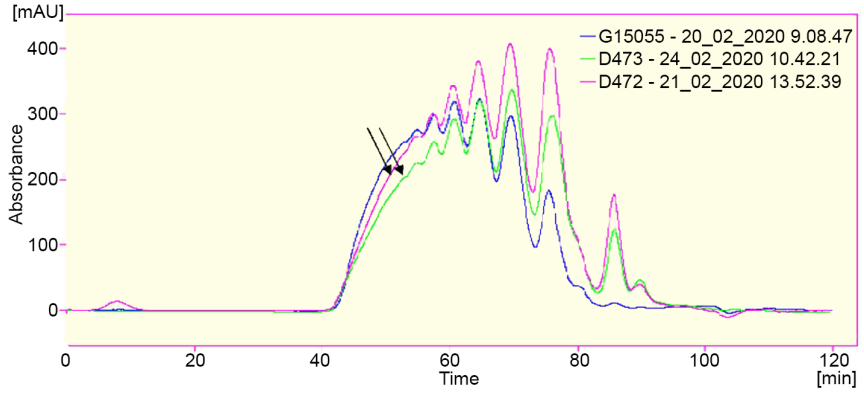


c

Fig. 2. Dynamics of changes in molecular weight distribution parameters in synthesized samples depending on temperature: *a* – Molecular Weight Distribution and Weight-Average Molecular Weight, Da; *b* – Low Molecular Weight Compounds (2000–8000 Da), %; *c* – High Molecular Weight Compounds (2000–8000 Da), %



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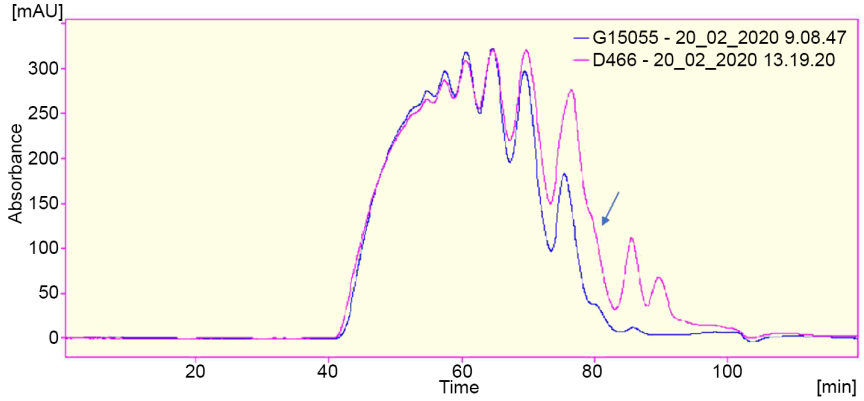

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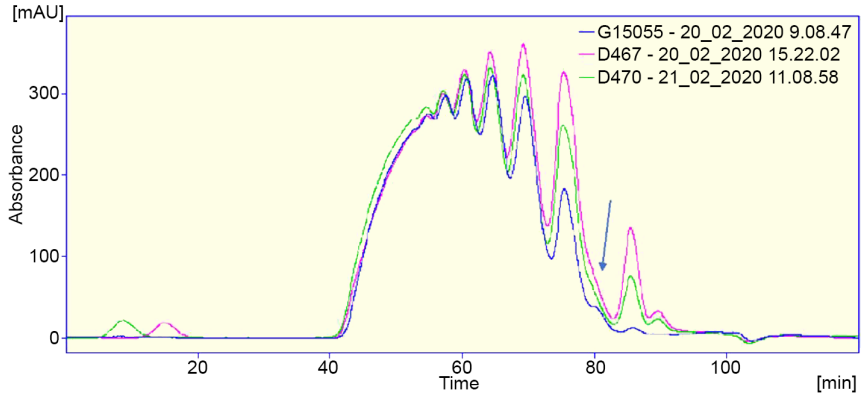

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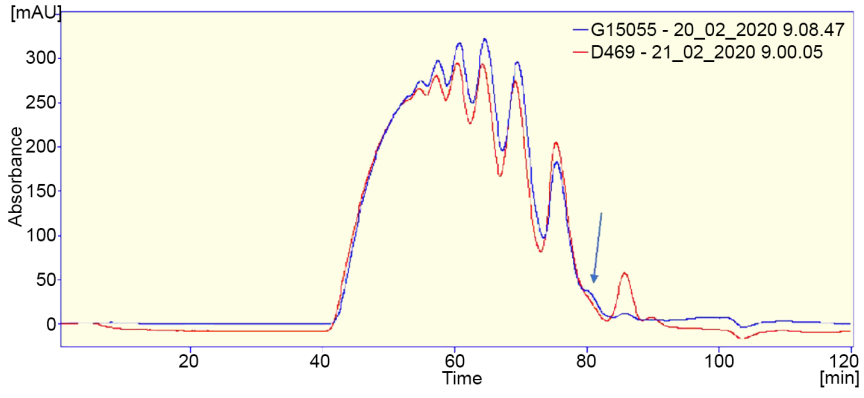


d

Fig. 3. Profiles of the distribution of oligosaccharide fractions of processed samples of crude Enoxaparin in comparison with Clexane: *a* – distribution of oligosaccharide fractions of Clexane (blue), D472 (pink), and D473 (green); *b* – distribution of oligosaccharide fractions of Clexane (blue) and D466 (pink); *c* – distribution of oligosaccharide fractions of Clexane (blue), D467 (pink) and D470 (green); *d* – distribution of oligosaccharide fractions of Clexane (blue) and D469 (red)

Table 1

The results of the analysis of samples of crude Enoxaparin sodium with variations in the amount of alkali and the temperature of the reaction mixture according to the specification of JSC Farmak (after purification with methanol)

NaOH/ester			0.06			0.07			0.08		
T (°C)			57	62	67	57	62	67	57	62	67
–			D469	D467	D472	D470	D466	D473	D471	D468	D474
Appearance	Powder or crystalls with yellow or almost yellow color		Yellow color powder								
pH	Info value		8.13	8.33	7.89	8.54	8.79	7.18	8.77	9.67	8.17
Loss on drying	Not more than 10 %		8.65	8.28	8.65	8.66	8.42	9.21	8.28	8.65	8.93
Residual solvents	Methanol	NMT 3000 ppm	22820	1280	13361	18809	275	323	14133	7965	12818
	Methylene chloride	NMT 600 ppm	0	0	0	0	0	0	0	0	0
Molecular Weight Distribution and Weight-Average Molecular Weight	MW	3800–5000	4140	3712	3335	4015	4124	3555	3725	3758	3413
	<2000 Da	12–20 %	18.5	24.6	30.5	21.1	19.0	26.7	24.9	24.6	28.5
	2000–8000 Da	68–82 %	73.9	70.2	66.3	71.7	73.2	69.0	69.7	69.8	68.0

Table 2

Results of the compositional analysis of samples of crude Enoxaparin sodium with variation of the amount of alkali and the temperature of the reaction mixture by the HSQC method (2D-NMR)

NaOH/ester	0.06			0.07			0.08			Clexane*	
T (°C)	57	62	67	57	62	67	57	62	67		
Amines	D469	D467	D472	D470	D466	D473	D471	D468	D474	Min	Max
ANS,6xaRed	10.9	11.9	12.8	9.6	8.4	10.6	8.8	8.8	8.9	7.8	9.0
ANS,6XbRed	1.4	1.5	1.6	1.2	0.9	1.2	1.1	1.1	1.0	1.0	1.2
ANAc,6xaRed	0.5	0.5	0.6	0.5	0.4	0.5	0.5	0.4	0.4	0.3	0.4
1,6anANS	1.2	1.6	1.6	1.9	3.3	2.6	2.8	3.2	3.6	2.0	2.3
1,6anMNS	1.4	1.8	1.9	2.1	3.9	2.9	3.0	3.6	4.0	2.4	2.5
MNS,6XaRed	3.0	3.1	3.3	3.3	2.4	2.7	2.9	2.2	2.2	2.6	3.0
%A6S	82.1	81.4	81.2	80.5	78.7	80.2	79.7	79.1	79.2	81.8	82.9
Uronic acid	D469	D467	D472	D470	D466	D473	D471	D468	D474	Min	Max
ΔU42S	18.1	20.2	21.9	19.2	20.4	21.1	20.3	20.3	21.7	17.3	18.1
ΔU4	1.6	1.9	2.1	1.8	1.9	1.9	1.9	2.0	2.2	1.1	1.2
Epox	0.9	0.9	0.8	1.2	1.3	1.1	1.5	2.2	1.4	0.2	0.6
GalA	2.1	1.7	1.9	1.8	1.8	1.7	1.8	1.7	1.9	1.2	1.8
ΔU42S/ΔU	11.0	10.7	10.2	10.7	10.7	11.0	10.5	10.3	9.9	15.1	15.7

Note: based on the analysis of the original Clexane from the database of the Ronzoni Institute

4. 2. 2. Variation of holding time

The following combination was determined as optimal conditions in the previous study: alkali/ester ratio 0.06 and holding temperature 57 °C, when studying the effect of variation of holding time, these indicators were constant. The holding time was increased to 1.5 hours, 2 and 3 hours. As can be seen from the Table 3, all processed samples under such conditions, regardless of the holding time, meet the requirements of the laboratory specification, formed on the basis of the EP monograph Enoxaparin sodium.

The analysis of the composition of the obtained samples (Table 4) and the comparison of the distribution of molecular weight (Fig. 4) was used as the basis for choosing the optimal time for holding the reaction mixture.

Table 3

The results of the analysis of samples of crude Enoxaparin sodium with variation of the time of holding of the reaction mixture according to the specification of JSC Farmak (after purification with methanol)

Time			1 h	1.5 h	2 h	3 h
Sample			D469	D484	D485	D524
Appearance	Powder or crystalls with yellow or almost yellow color		Yellow color powder			
pH	Info value		8.13	8.46	8.62	7.43
Loss on drying	Not more than 10%		8.65	4.22	5.20	9.97
Residual solvents	Methanol	NMT 3000 ppm	22820	517	427	0
	Methylene chloride	NMT 600 ppm	0	0	0	0
Identification (Molecular Weight Distribution and Weight-Average Molecular Weight)	MW	3800–5000	4140	4591	4626	4580
	<2000 Da	12–20 %	18.50	18.17	18.03	16.30
	2000–8000 Da	68–82 %	73.90	69.90	69.74	72.19

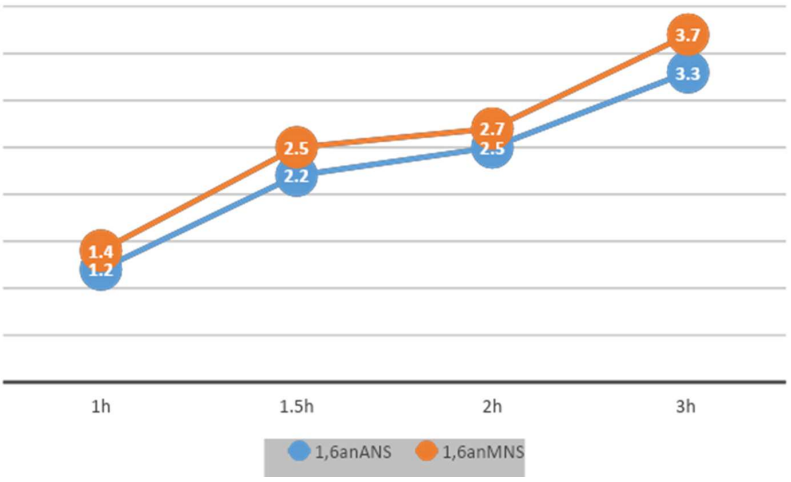
A comparative assessment of the dynamics of the content of 1,6-anhydrostructures 1,6anANS/MNS and ANS/MNS,6xaRed in the processed samples depending on the exposure time was carried out (Fig. 5).

Table 4

The results of the compositional analysis of samples of crude Enoxaparin sodium with variation of the time of holding of the reaction mixture (alkali/ester ratio 0.06, temperature 57 °C) by the HSQC method (2D-NMR)

Time	1 h	1.5 h	2 h	3 h	Clexane*	
–	D469	D484	D485	D524	Min	Max
Amines						
ANS,6xaRed	10.9	10.1	9.6	7.4	7.8	9.0
ANS,6XbRed	1.4	1.3	1.2	0.8	1.0	1.2
ANAc,6xaRed	0.5	0.6	0.6	0.3	0.3	0.4
1,6anANS	1.2	2.2	2.5	3.3	2.0	2.3
1,6anMNS	1.4	2.5	2.7	3.7	2.4	2.5
MNS,6XaRed	3.0	2.9	2.7	1.8	2.6	3.0
%A6S	82.1	80.4	80.0	80.3	81.8	82.9
Uronic acid						
ΔU42S	18.1	19.2	19.2	18.2	17.3	18.1
ΔU4	1.6	1.7	1.7	1.3	1.1	1.2
Epox	0.9	0.9	0.9	1.0	0.2	0.6
GalA	2.1	2.0	1.9	1.3	1.2	1.8
ΔU42S/ΔU	11.0	11.3	11.1	14.3	15.1	15.7

Note: based on the analysis of the original Clexane from the database of the Ronzoni Institute



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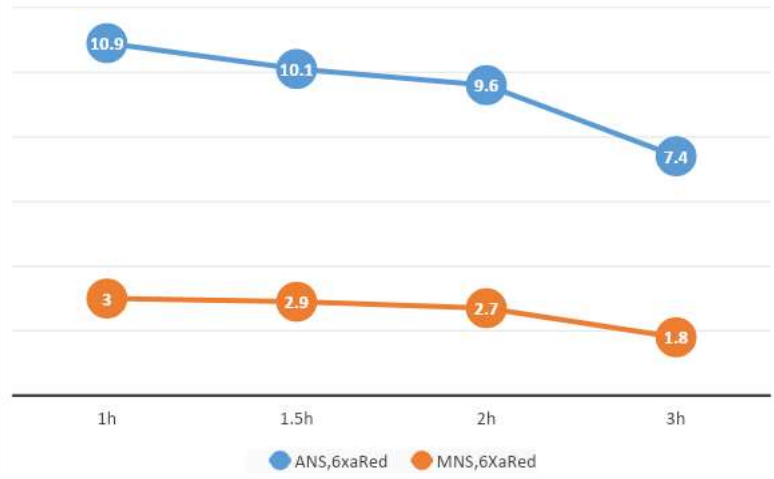

b

Fig. 5. Dynamics of changes in the content of 1,6anANS/MNS and ANS/MNS,6xaRed in crude Enoxaparin sodium depending on the holding (base/ester ratio 0.06, temperature 57 °C): *a* – dynamics of changes in the content of 1,6anANS/MNS; *b* – dynamics of changes in the content of ANS/MNS,6xaRed

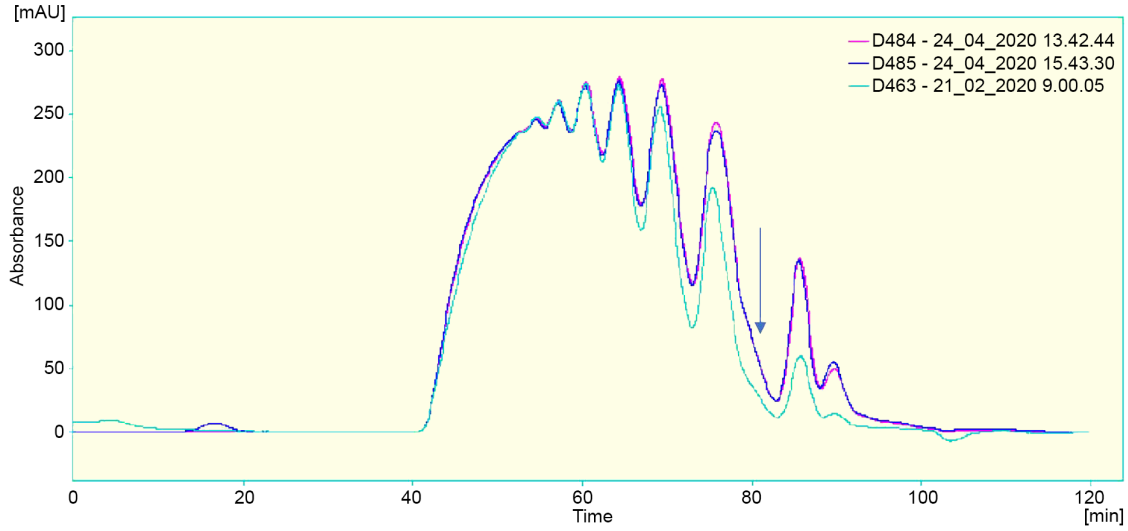

Fig. 4. Distribution of molecular weight in processed samples of crude Enoxaparin: D484 (pink), D485 (blue) and D469 (blue)

5. Discussion of research results

5. 1. Determination of "standard" synthesis conditions

The first stage of research was the "rough" selection of synthesis conditions. At this stage, we compared samples obtained under the synthesis conditions specified in the patent [22] and similar to (2) but with a reduced amount of alkali and holding time. These conditions were chosen on the basis of previous studies. When using the patent technique, significant depolymerization occurs, as a result of which the obtained product has a molecular weight lower than required. Reducing both the amount of alkali and temperature had a positive effect on the quality of the obtained product due to softening of the synthesis conditions. For further research and optimization under standard conditions, the alkali/ester ratio of 0.07, the temperature of 62 °C, and the holding time of 1 hour were chosen.

5. 2. Optimization of standard synthesis conditions

During studying variations of synthesis parameters, our task was to study not only compliance with pharmacopoeial quality requirements, but also establishing compliance with other criteria of equivalence to the originator. In particular, the analysis of the results of the study of the composition and determination of the distribution of oligosaccharide chains. Scientists use various methods for this. The most informative of them are the methods of two-dimensional NMR spectroscopy, size-exclusion chromatography (SEC) [6, 24]. Therefore, we used these methods to study the obtained samples. Having analyzed the compliance of the obtained samples with the requirements of the specification (Table 1), we decided to determine the general patterns of the influence of temperature for various alkali/ester ratios at a constant holding time on the identification parameters. As can be seen from Fig. 2, they can be characterized as follows:

– an increase in the temperature of the reaction mixture leads to a decrease in the molecular weight (Fig. 2, *a*);

– an increase in the temperature of the reaction mixture leads to an increase in the content of low molecular weight compounds (Fig. 2, *b*);

– an increase in the temperature of the reaction mixture leads to a decrease in the content of high-molecular compounds (Fig. 2, *c*).

These regularities are expected, since in general, an increase in temperature leads to an intensification of the depolymerization process. In addition, we analyzed the component composition of the obtained samples. D468, D471, D472, D473 and D474 are the samples that do not meet the requirements of EP in terms of molecular weight (Table 1). They are characterized by a high level of depolymerization, which is confirmed by the high content of residues at the reducing ends of the molecule – ANS/MNSred, 1.6anMNS/ANS and structures at the not-reducing ends of the molecule – ΔU42S, ΔU4 (Table 2). The D473 sample, which was processed at a higher temperature, contains a significantly lower number of high molecular weight oligomers compared to Clexane (Fig. 3, *a*) and correlates with the compositional analysis and the pharmacopoeial analysis of the molecular weight distribution. Under "standard" conditions without variations (D466), a fairly high level of depolymerization is also observed, as evidenced by the increased content of 1,6anANS, 1,6anMNS and low ΔU42S, ΔU4, respectively (Table 2). The analysis of the distribution of fractions shows a correspondence in the region of high-molecular fragments, while the region of low-molecular fragments is overestimated compared to the originator (Fig. 3, *b*). Samples D467, D469, D470 demonstrated a significantly lower content of 1,6anANS, 1,6anMNS residues at the reducing ends of the molecule, and the distribution of high molecular weight fractions was comparable to the originator (Table 2). With regard to low molecular weight fractions, the closest result to Clexane among these samples has sample D469 (Fig. 3, *c*, *d*). Summarizing the obtained profiles

of the distribution of fractions in the synthesized samples of crude Enoxaparin (Fig. 3), it can be argued that an increase in the amount of NaOH provokes a decrease in high-molecular oligosaccharides compared to Clexane, while a simultaneous increase in the temperature and amount of NaOH decreases the amount of high-molecular oligosaccharides and increases the amount of low-molecular ones, respectively.

According to the results of a complex analysis of the composition and distribution of oligomeric fractions, sample D469 is characterized by maximum similarity to the originator among the developed samples of Enoxaparin, thus the most acceptable for synthesis are the amount of alkali 0.6 g and the temperature 57 °C. An additional advantage of the synthesis at a lower temperature is compliance with the main postulates of "green chemistry" [25]. The slightly lower content of 1,6anANS, 1,6anMNS (Table 2) can be corrected by prolonging the reaction.

After studying the obtained samples and determining the closest to the original drug, the holding time was taken into consideration. As can be seen from Table 3, samples D484, D485 expectedly demonstrate an increased level of 1,6anANS, 1,6anMNS compared to D469, which allows us to assert the feasibility of such an increase in holding time (1.5 and 2 h, respectively). At the same time, in the sample D524 (holding time 3 hours), the composition of the molecule shifts towards an increased degree of depolymerization.

The profile of molecular weight distribution of samples D484 and D485 coincides with D469 in the area of distribution of high molecular weight fractions, however, an increase in the level of low molecular weight fractions is observed, which is explained by a higher degree of depolymerization (Fig. 4). When prolonging the holding time to 3 hours (D524, Table 4), an increase in the degree of depolymerization is observed, which correlates with the number of 1,6-anhydrostructures and an increase in signals in the area of low molecular weight fractions while maintaining high molecular weight fractions comparable to the originator.

As can be seen from the obtained experimental data (Fig. 5), with an increase in the holding time to 3 hours at constant temperature and alkali concentration, the number of 1,6-anhydro structures increases and the number of ANS/MNS,6xaRed at the reducing ends of the molecule decreases.

Thus, an interval of 1.5–2 hours can be considered the optimal time of holding. In samples of crude Enoxaparin sodium obtained under such conditions (D484 and D485), the composition is more balanced and as close as possible to the originator.

Study limitations. The limitation was that a larger variation of the parameters can negatively affect the characteristics of the Enoxaparin sodium substance. Also, increasing the variation of technological parameters during the development of synthesis technology usually requires additional resources in terms of personnel, materials and time.

Prospects for further research. As part of further research, it is planned to consider the issues of energy efficiency and waste minimization for greening the synthesis conditions. Chemical reagents and solvents used in the process must also be evaluated for their environmental impact. It is also planned to investigate and optimize the method of purification of crude Enoxaparin sodium.

6. Conclusions

The technological parameters of the key stage of Enoxaparin sodium synthesis – depolymerization of heparin benzyl ester – were experimentally determined. The specified parameters (alkali/benzyl heparin ester ratio 0.06; temperature – 57 °C, reaction mixture holding time – 1.5 hours) make it possible to obtain Enoxaparin sodium, which meets the requirements of the EP monograph. In addition, NMR spectroscopy and size-exclusion chromatography methods established that the composition of Enoxaparin sodium obtained under these conditions is balanced and as close as possible to the original drug Clexane®/Lovenox®. Thus, as a result of this study, the technological parameters of the synthesis of a sample of Enoxaparin sodium were determined, which satisfies at least three requirements of the FDA regarding the equivalence of generic drugs to the original.

Conflict of interests

The authors declare that they have no conflict of interest in relation to this research, including financial, personal, authorship or other nature, which could affect the research and its results presented in this article.

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Data availability

Data will be made available on reasonable request.

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Yuliia Bovsunovska*, Engineer-Technologist of Bioorganic Synthesis, JSC Farmak, Kyrylivska str., 63, Kyiv, Ukraine, 04080, Postgraduate Student, Department of Pharmaceutical Chemistry, National University of Pharmacy, Pushkinska str., 53, Kharkiv, Ukraine, 61002

Vitalii Rudiuk, Head of Laboratory, API Synthesis Laboratory, JSC Farmak, Kyrylivska str., 63, Kyiv, Ukraine, 04080
ORCID: https://orcid.org/0000-0003-3440-1139

Volodymyr Mishchenko, PhD, Associate Professor, Department of Pharmaceutical Technologies and Medicines Quality Assurance, Institute for Advanced Training of Pharmacy Specialists, National University of Pharmacy, Pushkinska str., 53, Kharkiv, Ukraine, 61002

Victoriya Georgiyants, Doctor of Pharmacy, Professor, Head of Department, Department of Pharmaceutical Chemistry, National University of Pharmacy, Pushkinska str., 53, Kharkiv, Ukraine, 61002

***Corresponding author:** Yuliia Bovsunovska, e-mail: juliabovsu@gmail.com*