chemistry proceedings

MDPI

Proceeding Paper

Depolymerization of heparin benzyl ester in a flow system †

Richard T. Taylor [1,*] and Rebecca G. Brunner [2]

[1] Miami University Oxford, OH USA; taylorrt@muohio.edu
[2] Miami University Oxford, OH USA; brunnerg@miamioh.edu
* Correspondence: taylorrt@muohio.edu
† Presented at the 26th International Electronic Conference on Synthetic Organic Chemistry; Available online: https://ecsoc-26.sciforum.net.

Abstract: The preparation of enoxaparin via the base promoted depolymerization of heparin is well known. In this paper we present our efforts to convert this preparation into a flow method. Streams of base and ester solutions are combined and heated in a flow reactor, Variations in flow rate, temperature and residence time were examined. NMR analysis demonstrates that careful control of the reaction parameters affords enoxaparin-like material. Analysis was carried out by NMR spectroscopy and optimum conditions determined.

Keywords: Flow processing; heparin; enoxaparin

Citation: Taylor, R.T.; Brunner, R.G. Depolymerization of heparin benzyl ester in a flow system. **2022**, *4*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Published: 15 November 2022

1. Introduction

Heparin is a highly charged, highly anionic glycoaminoglycan valued primarily for its anticoagulant properties [1]. In general, it is a linear polymer of alternating glucosamine and iduronic acid saccharides. In addition, heparin may include charged groups including sulfonates and carboxylates. The amino group in the glucosamine is generally observed as either a bare amino (NH_2), an acetamide ($NHCOCH_3$), or an aminosulfate ($NHSO_3^-$). The molecular weight is distributed about an average of around 15000 g/mol. Certain crucial pentasaccharide sequences along the chain are responsible for the anticoagulant activity of heparin as it binds to antithrombin. See Figure 1 for a generalized structure of heparin.

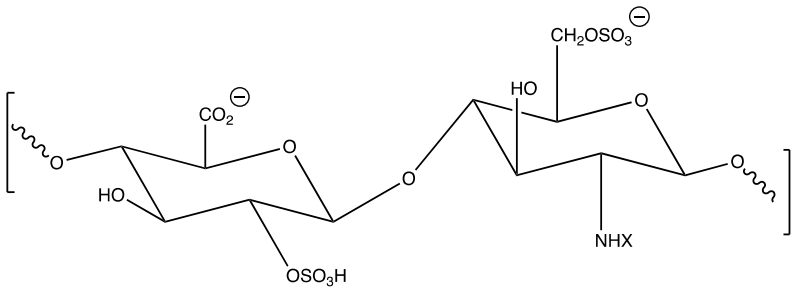

Figure 1. Generalized structure of heparin; amino variations as described above.

While unfractionated heparin remains a very important drug, fractionated or low molecular weight heparins (LMWH) were introduced as substitutes. These heparin derivatives are important in terms of clinical use due to reduced hemorrhagic side effects, more predictable pharmacological action, and improved bioavailability. Additionally, delivery of LMWHs is more convenient through subcutaneous administration [2].

One of the most commonly used LMWHs is enoxaparin with average molecular weight about 4500 [3]. Presently, enoxaparin is prepared in a batch process [4]. In a typical process, a benzyl ester of heparin is treated with base and moderate heat (55-65 °C) for

from 30 min to two hours with some post reaction processing to afford the LMWH. The structure of enoxaparin is shown in Figure 2, and can be characterized by the carbon-carbon double bond that forms at the non-reducing end. When produced, about 15-25% of the polysaccharide chains in enoxaparin have the 1,6 anhydro ring structure at the reducing end.

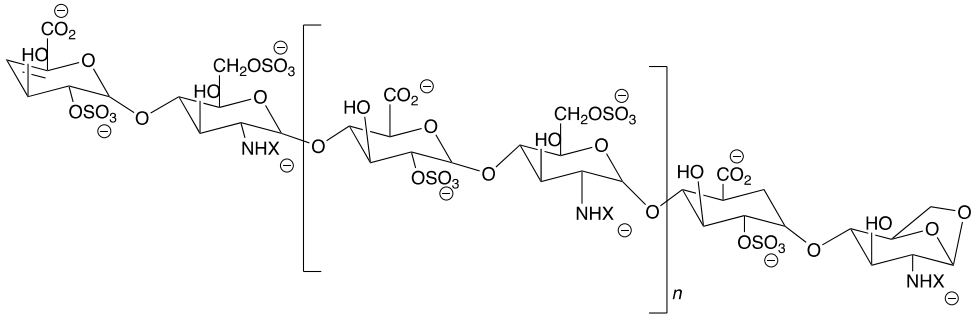

Figure 2. Generalized structure of enoxaparin.

The objective of our study was to investigate the base promoted depolymerization of heparin benzyl ester using a flow system. The potential advantages of a flow process have been well articulated and include reproducibility, safety, and quality control [5].

2. Methods and Materials

Heparin benzyl ester was provided by Smithfield Biosciences. All other reagents were used as received. NMR spectra were recorded on a Bruker Avance 500 MHz NMR instrument at 298K and D_2O as solvent.

Procedure for Flow Reactions

Continuous flow experiments were carried out on the Syrris FRX flow system with a 12.05 mL reactor coil (1/16" i.d. tubing). *Note: The continuous flow system was flushed with water before the reaction was run.* An aqueous solution of heparin benzyl ester and an aqueous solution of sodium hydroxide were merged through a t-mixer. The merged solution was pumped through the coil submerged in an oil bath at a regulated temperature. The reaction stream was collected in a flask and neutralized with dilute HCl. Subsequently, the product was diluted with 3X volume of methanol, the solution evaporated, washed with acetone, purified through cassette dialysis (slide-A-Lyzer G2, 2000 MWCO, Thermo-Fisher, Waltham, MA, USA), and freeze dried. HNMR was recorded on the recovered material.

The variations in reaction conditions are presented in Table 1.

Table 1. Reaction parameters for flow trials.3.1. Subsection.

Entry	Heparin Ester solution	NaOH solution	Flow ester (mL/min)	Flow base (mL/min]	T (°C)	Reaction Time (min)
1	2g/20mL	2g/20mL	0.25	0.25	70	24.1
2	2g/20mL	2g/20mL	0.12	0.12	50	50.2
3	2g/20mL	2g/20mL	0.12	0.12	65	50.2
4	2g/20mL	2g/20mL	0.12	0.12	75	50.2
5	2g/20mL	2g/20mL	0.12	0.12	65	50.2
6	2g/20mL	2g/20mL	0.24	0.24	65	25.1
7	2g/20mL	2g/20mL	0.12	0.14	55	50.2
8	2g/20mL	2g/20mL	0.24	0.24	75	25.1
9	4g/40mL	4g/40mL	0.24	0.24	65	25.1

10	4g/40mL	4g/40mL	0.24	0.24	55	25.1
11	0.65g/6.5mL	0.65g/6.5mL	0.48	0.48	55	12.6
12	1.0g/20mL	1.0g/20mL	0.24	0.24	55	25.1
13	1.5g/15mL	1.5g/15mL	0.48	0.48	55	12.6

3. Results

Our objective in this study was to derive flow conditions that gave rise to the most 'enoxaparin-like' product by HNMR. Our inspection of the results in the various entries concentrated on two peaks: The vinyl resonance at about 6.2ppm and the acetamide peak at about 2.2. As will be discussed below, the best match for USP enoxaparin as compared to a reaction product was for entry 12 of 25.1min residence time and 55C temperature. The stacked spectra are presented below in Figure 3.

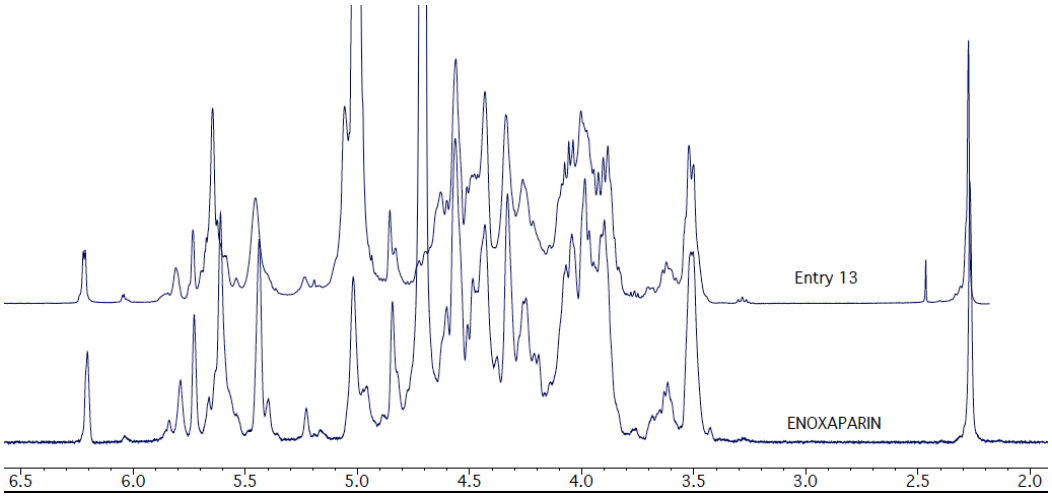

Figure 3. Stacked spectra of enoxaparin and the product derived under entry 13 conditions.

From this comparison, we have established that the crude reaction product using these conditions are similar to enoxaparin as refined and purified. The supplemental information includes the NMR spectra of other entries. Variations produce features similar to enoxaparin but the conditions described here are the optimum we have observed.

4. Discussion

The parameters we chose for our investigation were informed by the batch reaction data previously reported. There are two simultaneous reactions that must be balanced: saponification of the ester and elimination for the elimination process. If the saponification predominates, the molecular weight would be higher than desired. If the elimination predominates, the molecular weight would be low.

Figure 4 shows the HNMR spectrum of enoxaparin USP. The vinyl peak at 6.2 is new to the spectrum (compared to heparin sodium) and is a marker for the depolymerization extent while the NAc peak at 2.2 acts as a standard for the starting enoxaparin ester [6,7]. The balance of the signals is a good indicator of average molecular weight. Spectra of other entries of Table 1 are included in the supplemental materials. A smaller peak in the vinyl region is diagnostic for too high a molecular weight, while those at a higher temperature showed substantial formation of extraneous peaks [8].

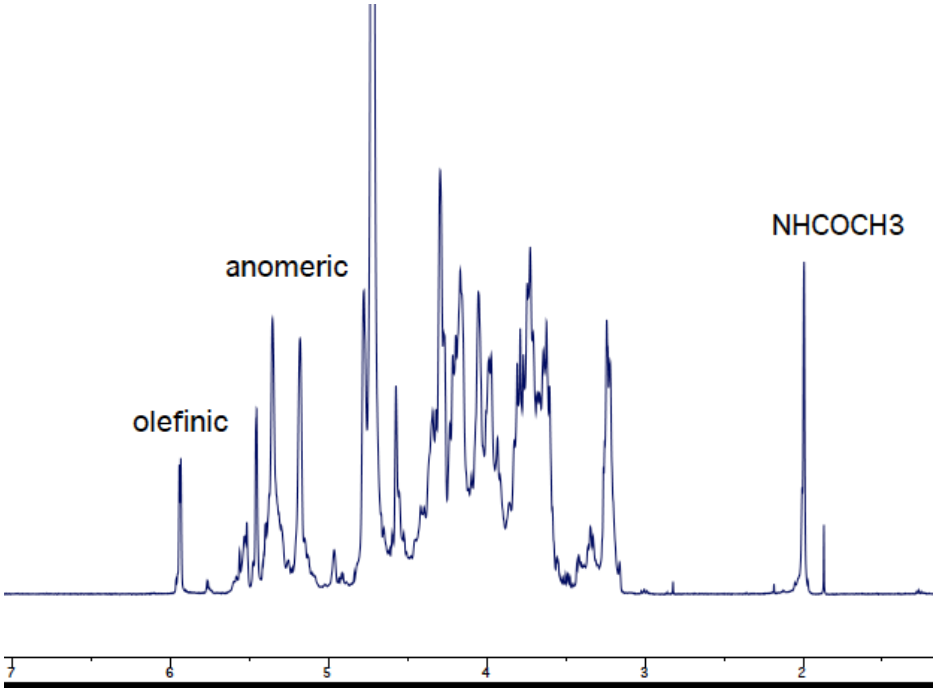

Figure 4. HNMR spectrum of enoxaparin USP.

5. Conclusion

The base promoted depolymerization of heparin benzyl ester can be conducted using a flow reactor. Conditions have been developed to produce materials that are similar to enoxaparin by NMR. Further work should be directed at elaborating the flow scheme to include refining of the crude material by a flow method for desalting and isolating the product.

6. Patents

The authors have filed a provisional patent application based on this work.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, NMR spectra of enoxaparin USP and selected entries from Table 1.

Funding: This research was funded by Smithfield Biosciences

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

Data Availability Statement: Data can be obtained from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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