Depolymerization of Heparin Benzyl Ester in a Flow System †

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† Presented at the 26th International Electronic Conference on Synthetic Organic Chemistry, 1–15 November 2022;
Available online: https://sciforum.net/event/ecsoc-26.

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, and the variations in flow rate, temperature, and residence time were examined. NMR analysis demonstrated that careful control of the reaction parameters affords enoxaparin-like materials. An analysis was carried out by NMR spectroscopy and the optimum conditions were determined.

Keywords: flow processing; heparin; enoxaparin

1. Introduction

Heparin is a highly charged, highly anionic glycosaminoglycan 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 at about an average of around 15,000 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.](image)

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 an 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 a base and moderate heat (55–65 °C) for from 30 min to 2 h 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.](image)

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 298 K and using D₂O as the 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 was 
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>
<thead>
<tr>
<th>Entry</th>
<th>Heparin Ester Solution</th>
<th>NaOH Solution</th>
<th>Flow of Ester (mL/min)</th>
<th>Flow of Base (mL/min)</th>
<th>T (°C)</th>
<th>Reaction Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 g/20 mL</td>
<td>2 g/20 mL</td>
<td>0.25</td>
<td>0.25</td>
<td>70</td>
<td>24.1</td>
</tr>
<tr>
<td>2</td>
<td>2 g/20 mL</td>
<td>2 g/20 mL</td>
<td>0.12</td>
<td>0.12</td>
<td>50</td>
<td>50.2</td>
</tr>
<tr>
<td>3</td>
<td>2 g/20 mL</td>
<td>2 g/20 mL</td>
<td>0.12</td>
<td>0.12</td>
<td>65</td>
<td>50.2</td>
</tr>
<tr>
<td>4</td>
<td>2 g/20 mL</td>
<td>2 g/20 mL</td>
<td>0.12</td>
<td>0.12</td>
<td>75</td>
<td>50.2</td>
</tr>
<tr>
<td>5</td>
<td>2 g/20 mL</td>
<td>2 g/20 mL</td>
<td>0.12</td>
<td>0.12</td>
<td>65</td>
<td>50.2</td>
</tr>
<tr>
<td>6</td>
<td>2 g/20 mL</td>
<td>2 g/20 mL</td>
<td>0.24</td>
<td>0.24</td>
<td>65</td>
<td>25.1</td>
</tr>
<tr>
<td>7</td>
<td>2 g/20 mL</td>
<td>2 g/20 mL</td>
<td>0.12</td>
<td>0.14</td>
<td>55</td>
<td>50.2</td>
</tr>
<tr>
<td>8</td>
<td>2 g/20 mL</td>
<td>2 g/20 mL</td>
<td>0.24</td>
<td>0.24</td>
<td>75</td>
<td>25.1</td>
</tr>
<tr>
<td>9</td>
<td>4 g/40 mL</td>
<td>4 g/40 mL</td>
<td>0.24</td>
<td>0.24</td>
<td>65</td>
<td>25.1</td>
</tr>
</tbody>
</table>
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 6.2 ppm 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 with 25.1 min residence time and 55 °C 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 when refined and purified. The supplemental information includes the NMR spectra of the other entries. The variations produced features similar to enoxaparin but the conditions described here are the optimal one that we observed.

4. Discussion

The parameters we chose for our investigation were informed by the batch reaction data that was previously reported. There are two simultaneous reactions that must be balanced: saponification of the ester and elimination. 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 USP enoxaparin. 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 the average molecular weight. The spectra of the other entries in Table 1 are included in the Supplementary 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].

<table>
<thead>
<tr>
<th>Entry</th>
<th>Heparin Ester Solution</th>
<th>NaOH Solution</th>
<th>Flow of Ester (mL/min)</th>
<th>Flow of Base (mL/min)</th>
<th>T (°C)</th>
<th>Reaction Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4 g/40 mL</td>
<td>4 g/40 mL</td>
<td>0.24</td>
<td>0.24</td>
<td>55</td>
<td>25.1</td>
</tr>
<tr>
<td>11</td>
<td>0.65 g/6.5 mL</td>
<td>0.65 g/6.5 mL</td>
<td>0.48</td>
<td>0.48</td>
<td>55</td>
<td>12.6</td>
</tr>
<tr>
<td>12</td>
<td>1.0 g/20 mL</td>
<td>1.0 g/20 mL</td>
<td>0.24</td>
<td>0.24</td>
<td>55</td>
<td>25.1</td>
</tr>
<tr>
<td>13</td>
<td>1.5 g/15 mL</td>
<td>1.5 g/15 mL</td>
<td>0.48</td>
<td>0.48</td>
<td>55</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Table 1. Cont.
5. Conclusions

The base-promoted depolymerization of heparin benzyl ester can be conducted using a flow reactor. The 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: https://www.mdpi.com/article/10.3390/ecsoc-26-13718/s1, NMR spectra of enoxaparin USP and selected entries from Table 1.

Author Contributions: R.T.T. authored the manuscript and directed the project. R.G.B. developed the methodology. All authors have read and agreed to the published version of the manuscript.

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.

References


