

# Evaluating the Biological Activity of Synthetic Heparin Analogues

Evangeline J.E. Chai<sup>1</sup>, Yongmei Xu<sup>2</sup>, Jian Liu<sup>2</sup>

<sup>1</sup>UCL School of Pharmacy, 29-39 Brunswick Square, London WC1N 1AX, UK

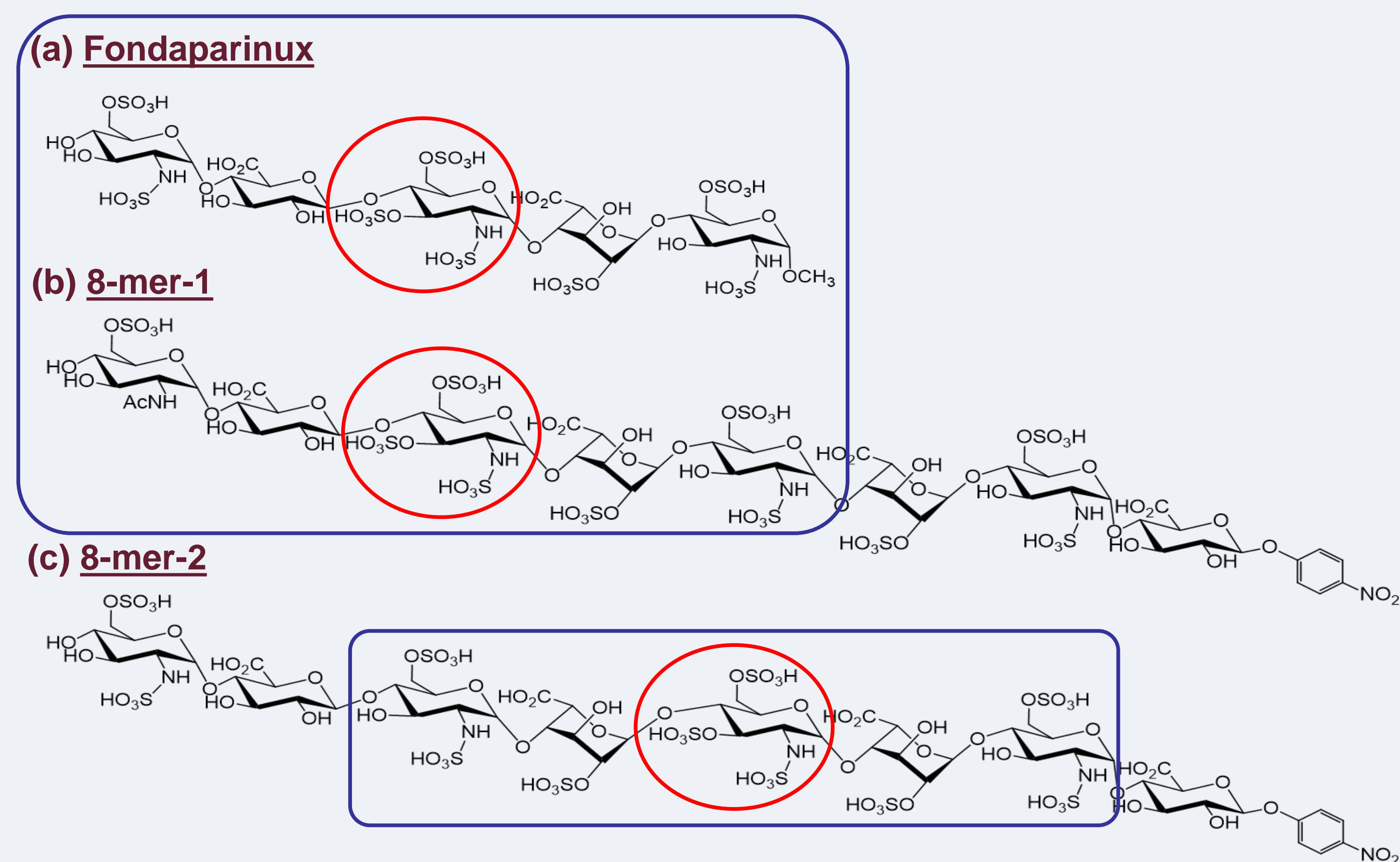
<sup>2</sup>Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA



## INTRODUCTION

Heparin is a medicinal product used to prevent and treat thrombotic disorders. It consists of disaccharide repeating units of an iduronic acid (IdoA) or glucuronic acid (GlcA) residue linked to a glucosamine (GlcN) residue, each of which may be sulfated<sup>1</sup>. This allows it to function as a potent cofactor of antithrombin (AT) in the inhibition of several coagulation enzymes including factor Xa, preventing the formation of a fibrin clot. A specific pentasaccharide sequence is required for AT binding to occur; the 3-O-sulfated glucosamine residue (GlcNS3S±6S), in particular, is essential<sup>2</sup>.

There are three groups of heparin products approved by the MHRA for clinical use: unfractionated heparin (UFH), low molecular weight heparins (LMWH) and fondaparinux. UFH and LMWHs are heterogeneous mixtures derived from animal sources, making them difficult to standardise and control for quality<sup>3</sup>. Fondaparinux is a fully synthetic compound containing the pentasaccharide AT-binding domain, but its long chemical synthesis requires 50 steps and has a low overall yield of ~0.1%<sup>5</sup>.



**Figure 1. Chemical structures of (a) fondaparinux (GlcNS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S, MW = 1508.2 Da), (b) 8-mer-1 (GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA-pnp, MW = 2330.8 Da) and (c) 8-mer-2 (GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-pnp, MW = 2368.9 Da).** The pentasaccharide AT-binding site of these compounds is highlighted in a box, and the key residue **GlcNS3S6S** is circled in red.

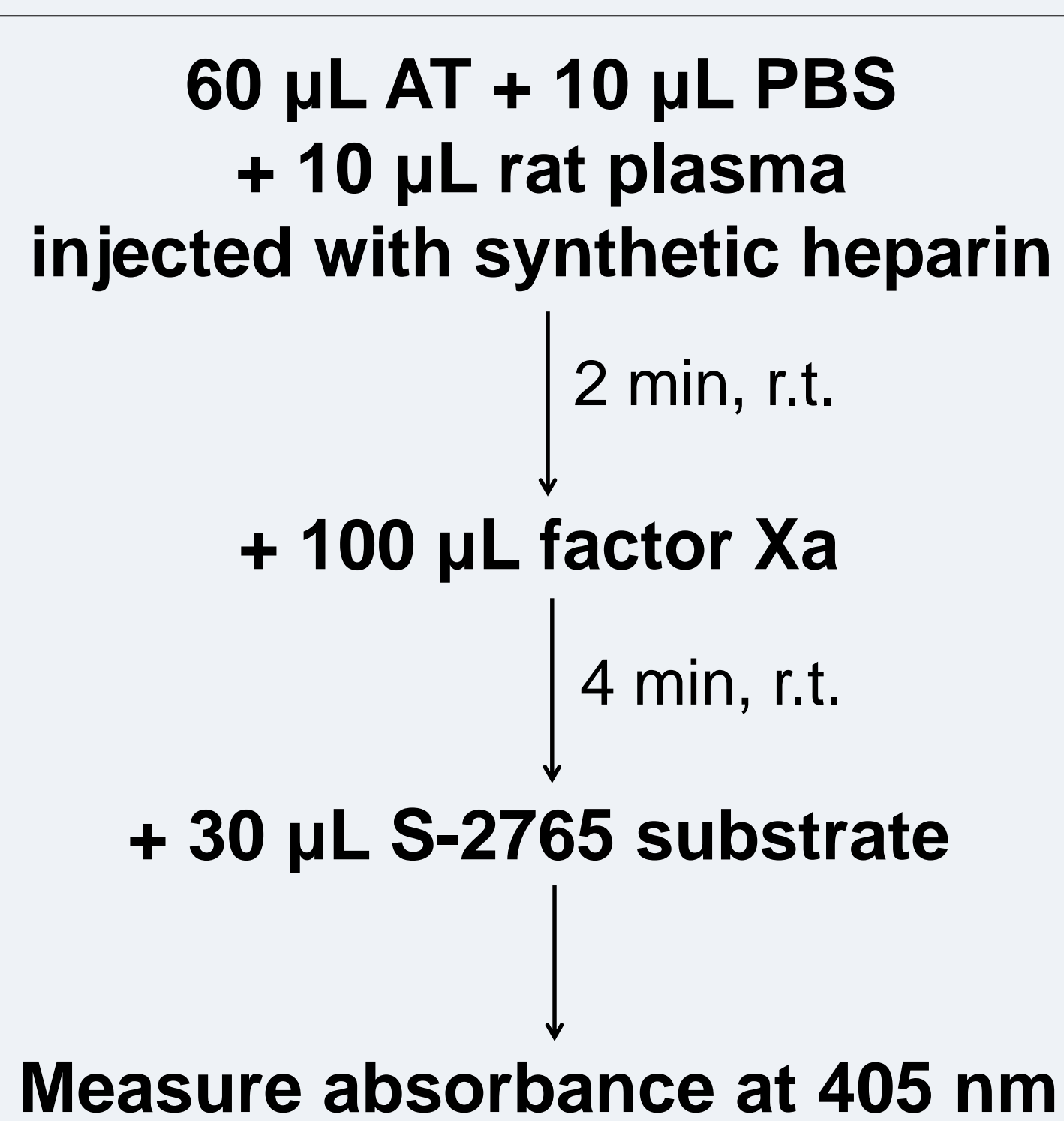
A recently-developed chemoenzymatic method, which mimics the biosynthetic pathway of heparin, offers a more efficient way of synthesising heparin analogues<sup>6</sup>. In this study, we sought to evaluate the *ex vivo* anticoagulant activity of two chemoenzymatically synthesised octasaccharide heparin analogues (8-mer-1 and 8-mer-2) as compared to fondaparinux (see Figure 1), to assess their feasibility of development as new drug candidates.

## METHODS

Sixteen 400-g Lewis rats were equally divided into four groups. Each group was intravenously administered with either 0.7mg/kg of fondaparinux, 1mg/kg of 8-mer-1 or 8-mer-2, or saline. Blood was then drawn at specified time points up to 8 hours after the compounds had been injected.

Plasma obtained from these blood samples were tested for their anti-factor Xa activity based on a previously published method<sup>7,8</sup> (see Scheme 1). The absorbance values were plotted against reaction time to measure the initial reaction rate for each sample, which was then used to calculate factor Xa activity.

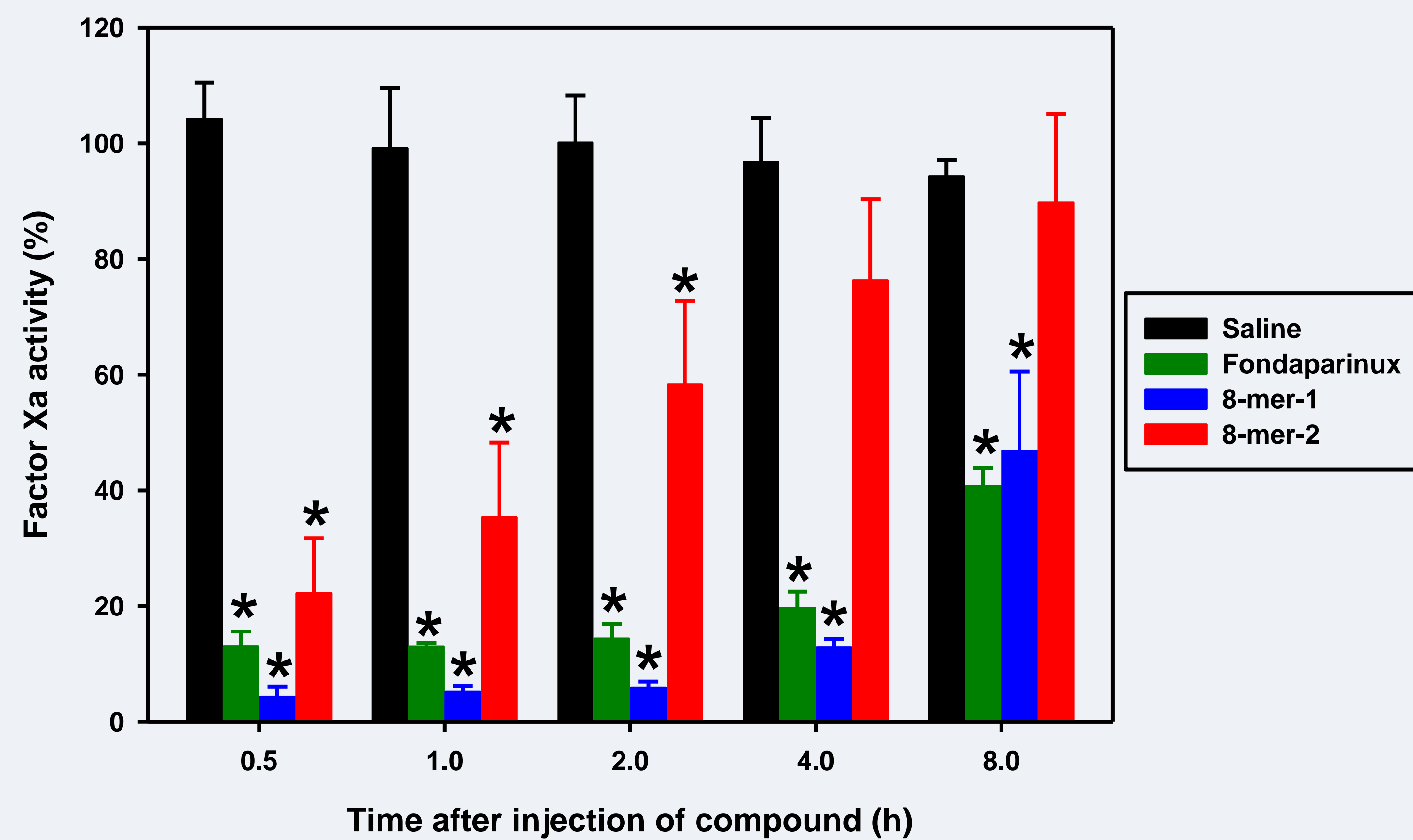
Statistical analyses were performed on factor Xa activity percentages for each time point. A single-factor ANOVA took all four experimental groups into account, while a two-tailed Student's t-test was conducted between the saline control group and each of the other three experimental groups.



**Scheme 1. Anti-factor Xa assay.** r.t. = room temperature, approximately 24°C.

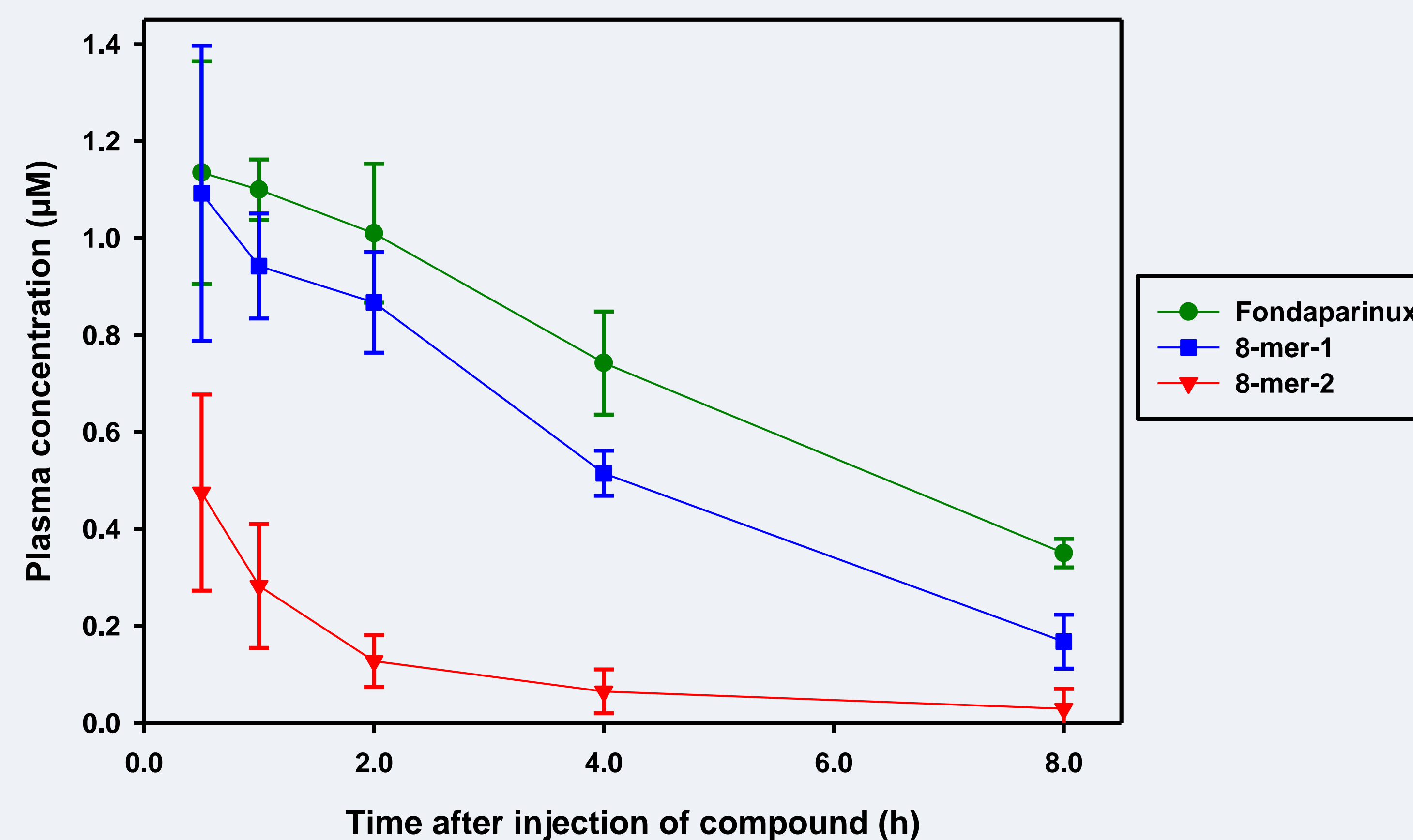
## RESULTS

We found that all three compounds inhibited factor Xa in the presence of AT (see Figure 2). Xa activity was significantly reduced ( $p < 0.05$ ) for fondaparinux and 8-mer-1 compared with saline at all time points; however, there was no significant difference in Xa activity between 8-mer-2 and saline beyond 4 hours after compound injection.



**Figure 2. Factor Xa activity of plasma samples from Lewis rats injected with test compounds.** \* indicates a significant difference ( $p < 0.05$ ) in Xa activity between that experimental group and the saline control group for that particular time point.

Using a calibration curve, the concentration of compound in the reaction mixture was extrapolated from each corresponding factor Xa activity percentage. This concentration was multiplied by a factor of 21 to obtain the concentration of compound in the rat plasma.



**Figure 3. Pharmacokinetic curves of ● fondaparinux ( $t_{1/2} = 4.42$  h), ■ 8-mer-1 ( $t_{1/2} = 2.77$  h) and ▼ 8-mer-2 ( $t_{1/2} = 2.82$  h).**

From the pharmacokinetic curves (see Figure 3), it appears that fondaparinux and 8-mer-1 are eliminated in a similar linear fashion, while 8-mer-2 appears to be cleared much faster at the early time points. 8-mer-2 is likely to follow a two-phase, two-compartment model of drug distribution, in which the pharmacokinetic profile becomes more linear after equilibrium has been established between the central and tissue compartments.

These differences between the three compounds may be linked to their chemical structures. The GlcA residue in the pentasaccharide AT-binding domain present in 8-mer-1 and fondaparinux has been replaced with IdoA2S in 8-mer-2. This change was previously thought to abolish anticoagulant effect in oligosaccharides, but has now been demonstrated to retain anti-factor Xa activity in 8-mer-2, and may be responsible for the difference in the pharmacokinetic profiles of both synthetic octasaccharides in spite of their structural similarities.

## CONCLUSIONS

- AT-binding ability and factor Xa inhibition is retained when GlcA is substituted for IdoA2S in the pentasaccharide AT-binding domain.
- This set of results validates the use of the chemoenzymatic method in developing a new generation of synthetic heparin analogues with desirable clinical properties and lower contamination risk compared with animal-sourced heparin.

**Abbreviations:** ANOVA: analysis of variance; AT, antithrombin; GlcA, glucuronic acid; GlcNAc6S, 6-O-sulfo N-acetylglucosamine; GlcNS6S, N,6-O-sulfo glucosamine; GlcNS3S6S, N,3,6-O-sulfo glucosamine; IdoA2S, 2-O-sulfo iduronic acid; LMWH, low molecular weight heparin; MHRA, Medicines and Healthcare Products Regulatory Agency; pnp, para-nitrophenyl; UFH, unfractionated heparin; Xa, activated factor X.

**References:**  
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