Short communication

Development of a method to analyze the complexes of enoxaparin and platelet factor 4 with size-exclusion chromatography

Fangxia Wu, Kai Dong, Meng Zhu, Qinghua Zhang, Bingying Xie, Duxin Li, Hao Gan, Robert J. Linhardt, Zhenqing Zhang

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A bstract

Heparin, a highly sulfated glycosaminoglycan, has been used as a clinical anticoagulant over 80 years. However, heparin-induced thrombocytopenia and thrombosis (HITT) is a serious side effect of heparin therapy, resulting in relatively high risk of amputation and even death. HITT is caused by forming of complexes between heparin and platelet factor 4 (PF4). Enoxaparin, one of the most commonly used low molecular weight heparin (LMWH), were developed in 1980’s. The lower molecular weight of enoxaparin reduces the risk of HITT by binding to less PF4. To detect the binding capacity between enoxaparin and PF4 could be an effect way to control this risk before it goes to patients. In this work, a size exclusion chromatography (SEC) method was developed to analyze the patterns of complexes formed between PF4 and enoxaparin. The chromatographic condition was optimized to separate PF4, enoxaparin, ultra-large complexes and small complexes. The linearity and stability of this method were confirmed. The impacts of PF4/enoxaparin mixture ratios and incubation time on the forming complexes were investigated. Four enoxaparin samples were analyzed with this method to verify its practicability. It is a robust, accurate and practicable method, and provides an easy way to monitor the capacity of enoxaparin forming complexes with PF4, suggesting the HITT related quality of enoxaparin.

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1. Introduction

Heparin, a highly sulfated glycosaminoglycan, has been used as a clinical anticoagulant over 80 years. It is one of the oldest drugs still used in clinic; it is one of the most complicated drugs with big and polydisperse molecular weight (MW) [1,2]. Heparin-induced thrombocytopenia and thrombosis (HITT) is a serious side effect of heparin therapy. HITT is developed in approximately 3%–5% of patients treated with heparin in 5–10 days. About 40%–75% of HITT patients appear arterial or venous thrombosis to varying degrees, resulting in relatively high risk of amputation and even death [3]. The forming of complexes between heparin and platelet factor 4 (PF4) is the major inducement for HITT [4]. PF4 is a lysine-rich tetrameric protein with a MW of 7.8 KDa, also known as CXCL4. It is stored in platelet alpha granules under normal physiological conditions. It releases into blood to gather platelets and accelerate coagulation when platelets are stimulated or activated, such as surgical trauma. Heparin, a negatively charged long-chain polysaccharide, is often used in surgery as anticoagulant. It can combine with a number of released positively charged PF4 [5–7]. Once the complexes of heparin/PF4 forms, B cell is easy to be stimulated and produce IgG. Subsequently, IgGs form antigenic complexes with the complexes of heparin/PF4. Then, the complexes of IgG/heparin/PF4 gather many platelets to aggregate in vessels. On one hand, the complexes aggregate too many platelets and induce macrophages to clear them, resulting in platelet decreasing in blood. On the other hand, the thrombocytopenia induces compensatory increasing of platelets and coagulation factors locally and results in serious local venous or arterial thrombosis [8,9].

To decrease the forming of heparin/PF4 complex is one of the most important ways to reduce HITT [10]. Low molecular weight heparins (LMWHs) have been developed since 1980’s. Enoxaparin is the most common LMWH widely used in clinic. The shorter sugar chains in LMWHs comparing to those in heparin have lower possibility to form the complexes with PF4 [11,12]. It was reported that the risk of HITT in patients treated with LMWH is 1%, which is
much lower than that of heparin. Furthermore, to detect the binding capacity between LMWH and PF4 could be an effect way to control this risk before it goes to patients.

In 1990s, mass spectrometry (MS) was used to characterize the non-covalent complex of heparin oligosaccharides and polypeptides, but it was hard to analyze those complexes quantitatively and the energy of ionization would affect the binding of those complexes differently [13,14]. A few methods have been developed to analyze the complexes of LMWHs/PF4 quantitatively. LMWH combining a large number of PF4 forms ultra-large complex of LMWH/PF4 (ULC); LMWH, combining relatively small number of PF4 forms small complex (SC). The particle size and distribution of ULCs and SCs can be investigated with electron microscope or photon correlation spectroscopy [15–17]. Furthermore, the quantitative analysis of ULC and SC was reported with radioisotope labeled chromatography [15]. However, those are not commonly used methods, or lack accuracy and precision. Thus, it is important to develop a practicable method to control the forming of LMWH/PF4 complexes.

In this study, a high performance size exclusion chromatography was developed to analyze the forming of enoxaparin/PF4 complexes. Three batches of enoxaparin were used to verify the method.

2. Materials and methods

2.1. Materials

PF4 was purchased from Sigma Chemical (St. Louis, MO). Enoxaparin standard was obtained from the USP (Rockville, MD). Three batches of enoxaparin were kindly provided by Hubei Enoray Biopharmaceutical Co., LTD. High-purity water (resistivity ≥18.2 Ω cm, 25 °C) was used throughout the study. All other chemicals and reagents were of HPLC grade.

2.2. Complex analysis with size exclusion chromatography (SEC)

Proper amount of PF4 was dissolved in phosphate buffer (pH 7.0; 10 mM) affording to 1 mg/mL solution. Different amounts of enoxaparin were dissolved in the 10 mM phosphate buffer affording to solutions with concentrations of 0.675, 1.25, 2.5 and 5 mg/mL. The ratios of enoxaparin and PF4 needs to be investigated as the different ratios would affect the forming of enoxaparin/PF4 complexes. In each experiment, 4 μL PF4 solution was mixed with 2 μL enoxaparin solution and incubated at 37 °C for 30 min before analysis. The analysis was performed on an Agilent 1290 system equipped with dual pumps (Agilent, CA, USA). A TSK gel G5000PWXL (10 μm, 7.8 mm × 300 mm) column was operated at 0.6 mL/min and 37 °C with isocratic gradient. The mobile phase is as same as the incubation phosphate buffer (pH 7.0; 10 mM). UV 232 nm was selected as the detection.

2.3. The impact of incubation time on the forming of complex

The enoxaparin/PF4 complexes could be generated in short time. While, the aggregated ULC could dissociate to SC, and SC could dissociate to unbound enoxaparin and PF4, when they incubate too long time [15]. So it is necessary to investigate the impact of incubation time on the forming of complex. The aliquots were taken at 0.5, 2 and 6 h.

![Fig. 1. SEC chromatograms of PF4 and enoxaparin, and their linearity.](image)

3. Result

3.1. Optimization of chromatographic conditions

Both PF4 and enoxaparin are polar macromolecules. They have different MW. Size exclusion chromatography (SEC) is the best way to separate them. Several SEC columns were used to separate PF4 and enoxaparin, including ACQUITY UPLC® BEH 200 Å SEC (Waters), ACQUITY UPLC® BEH 125 Å SEC (Waters), TSK G3000, 4000 and 5000 PWXL (TSK). The particle sizes of the first two columns are too small [18,19]. The peak of enoxaparin on these two columns was real wide and oligosaccharides with different sizes in enoxaparin were separated with these two columns. The size exclusion ranges of TSK 3000 and 4000 PWXL are too small for this work. The retention time of enoxaparin is close to the void volume. There is little room for these two column to optimize. It would be hard to separate ULC, SC, enoxaparin and PF4. TSK 5000 PWXL was selected as the column to do this work. To minimize the impact from separation process on the formed enoxaparin/PF4 complexes, the eluent was as same as incubation buffer, the operation temperature is as same as incubation temperature (37 °C).

Fig. 1 are the chromatograms of PF4 and enoxaparin. As PF4 has no specific UV absorption based on the UV spectrum (sFig. 1A), non-specific UV absorption wavelength was set at 210 nm to detect PF4. PF4 was eluted out from the column at about 19 min (Fig. 1A). Enoxaparin has a double bond on the uronic acid at the non-reducing end of the sugar chain. [20] It conjugates with the carboxyl group on the uronic acid. The UV spectrum (sFig. 1B) showed the specific UV absorption wavelength at 232 nm, which was used as enoxaparin detection wavelength in this work. The peak of enoxaparin was observed at 16.6 min (Fig. 1B). The MW of PF4 and enoxaparin are 7.8 and 4.5 kDa, respectively. However, the enoxaparin was eluted from SEC column earlier than PF4. It is believed that the linear shape of glycan in enoxaparin contributed to its performance in SEC. It is the main reason that smaller but linear enoxaparin eluted earlier than the bigger but globular PF4 in SEC. The peak areas of PF4 and enoxaparin were proportion to their
amounts. They showed good linearity in 1–10 μg and 0.1–20 μg, respectively (Fig. 1C and D).

3.2. The impact of the enoxaparin/PF4 ratio on the formation of complex

The impact of different PF4/enoxaparin ratio on the formation of complex were investigated. Enoxaparin with different concentrations were mixed with 1 mg/mL PF4 according to PF4/enoxaparin ratios: 0.5, 1:1, 1:2, and 1:4. They were all incubated for 30 min at 37 °C before analysis. Fig. 2 are chromatograms (232 nm) of samples with different PF4/enoxaparin ratios. The peaks corresponding to enoxaparin and PF4 were observed, but no peak corresponding to complex was observed in the chromatogram (Fig. 2A), when small amount of enoxaparin was mixed with PF4 (PF4/enoxaparin = 1:0.5). Two more peaks were observed at ~9.0 and 15.0 min other than the enoxaparin and PF4 peaks (Fig. 2B), when PF4/enoxaparin ratio is 1:1. These two peaks correspond to ULC and SC, respectively. The peak corresponding to ULC is much bigger than that of SC. The peak of ULC disappeared and the peak of SC increased (Fig. 2C), when more enoxaparin was mixed with PF4 (PF4/enoxaparin = 1:2). Enoxaparin peak increased a little more in Fig. 2D, when more enoxaparin kept adding (PF4/enoxaparin = 1:4). Thus, it could be imaged that the complex cannot be formed when enoxaparin's concentration is too low; multiple moles of PF4 can bind to single mole of enoxaparin and form ULC when enoxaparin’s concentration increases properly; multiple moles of PF4 have chances to bind to multiple mole of enoxaparin and forms more SC when enoxaparin's concentration is high enough. There are most patterns of complexes when PF4 and enoxaparin were mixed by the ratio of 1:1.

3.3. The impact of incubation time on the formation of complex

Same molar amount of enoxaparin and PF4 were mixed and incubated at 37 °C. The aliquots were taken at 0.5, 2 and 6 h for SEC analysis. The peaks corresponding to ULC, SC and free enoxaparin were integrated in the chromatograms (Fig. 3). The ratios of ULC/SC/enoxaparin in these chromatograms were calculated. No significant difference were observed between aliquots taken at 0.5 and 2 h. The ratios of ULC/SC/enoxaparin are 37:8:55 and 37:6:57 (Fig. 3A and B), respectively. The peak of ULC in the chromatogram of the aliquot taken at 6 h decreased significantly, and the ratio of ULC/SC/enoxaparin was measured at 8:15:77 (Fig. 3C), suggesting ULC dissociates after 2 h incubation. Thus, the patterns of complexes stay same when the mixture of PF4 and enoxaparin incubated at 37 °C less than 2 h, and the ULC dissociates afterwards.

3.4. Method stability

Five replicates were incubated at 37 °C for 0.5 h before SEC analysis. All experiments were performed on the PF4/enoxaparin ratio at 1:1. The ratio of peaks corresponding to ULC, SC and enoxaparin were calculated. The profiles of these chromatograms are same and the ULC/SC/enoxaparin ratios in the replicates are same too (Fig. 4). Thus, the method developed in this work is stable to analyze the complex patterns of PF4 and enoxaparin.

3.5. Consistency evaluation of different enoxaparins with current method

A USP enoxaparin and three batches of enoxaparin provided by Hubei Enoray Biopharmaceutical Co., LTD were analyzed with this method. All samples were mixed with same amount of PF4 (1:1) and incubated at 37 °C for 0.5 h. Each incubated sample was injected to SEC column right after incubation. The chromatography were performed at 37 °C too and the eluent is same as the incubation buffer. Four chromatograms were shown in Fig. 5. The ratios of ULC/SC/enoxaparin were measured based on the peak areas. No
significant difference of the PF4/enoxaparin complex patterns was observed between these samples.

4. Discussion and conclusion

A practicable method was developed in this work to investigate the PF4/enoxaparin complex patterns. The ratio of PF4 and enoxaparin was selected as 1:1, under which they form both ULC and SC. The incubation time was also investigated. It is proper to incubate PF4 and enoxaparin mixture at 37 °C for 0.5 h before SEC analysis. The ULC would disaggregate if the mixture incubated too long, such as 6 h. The SEC method separates 2 populations of enoxaparin/PF4 complexes (ULC and SC), free enoxaparin and PF4, which provides semi-quantitative data for comparison and accurate statement of the forming complexes. It is a robust, accurate and practicable method, and provides an easy way to monitor the capacity of enoxaparin forming complexes with PF4, suggesting the HITT related quality of enoxaparin.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.11.018.

References


![Fig. 5. Chromatograms of PF4 mixed with USP enoxaparin and three batches of enoxaparins, respectively.](image-url)