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Evaluating Heparin Products for Heparin-Induced Thrombocytopenia Using Surface Plasmon Resonance

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ABSTRACT

Heparin-induced thrombocytopenia (HIT) is an adverse immunological disorder caused by antibodies to platelet factor 4 (PF4)-heparin complexes. The analysis of HIT potential for different heparin and heparin-related products is important prior to their clinical application. Here, we report a rapid method for the evaluation of HIT potential of various heparin and heparin-related compounds using surface plasmon resonance (SPR). Solution competition between surface-immobilized heparin and soluble unfractionated heparin, low molecular weight heparin (LMWH), or ultra-LMWH binding to PF4 was performed using SPR to measure the half maximal inhibitory concentration (IC_{50}) of different heparin products. The IC_{50} values of different unfractionated heparin active pharmaceutical ingredients (APIs) varied from 0.38 to 0.6 μg/mL and the IC_{50} values of different LMWH APIs ranged from 2.4 to 2.9 μg/mL. The IC_{50} of Arixtra® (a synthetic pentasaccharide ultra-LMWH) was not measurable even at very high concentrations. These differences in IC_{50} values for different heparin products suggest a quantitative means for evaluating the HIT potential of various heparins and heparin-related products.

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Introduction

Heparin is the most widely used anticoagulant drug with more than 100 tonnes of heparin used annually,[1] with a projected global market to reach approximately $16.3 billion by 2025.[2] Heparin is a naturally occurring glycosaminoglycan, and pharmaceutical heparin is currently extracted primarily from porcine intestine but can also be prepared from bovine lung and intestine and ovine intestine.[3] Crude heparin is extracted from tissues collected in slaughterhouses, followed by its purification and processing to unfractionated heparin (UFH) and low molecular weight heparin (LMWH), manufactured under current good manufacturing conditions (cGMP) conditions.[4] Recent efforts on developing bioequivalent heparin and LMWH from non-animal sources have led to chemoenzymatic routes of heparin synthesis with a goal of addressing the sameness to reference listed drug (RLD), including chemical structure, biological activity, and immunogenicity.[5-7]

Heparin is a negatively charged linear glycosaminoglycan and is composed of alternating D-glucosamine and L-iduronic acid or D-glucuronic acid sugar residues that are highly sulfated (2.0-2.5 sulfate groups per disaccharide unit).[8] The interaction of heparin with proteins in a domain-specific or charged-dependent manner.[9] Heparin products have a highly sulfated pentasaccharide that binds to antithrombin III (AT) leading to inhibition of the blood coagulation cascade resulting in anticoagulation.[1,8] In addition to binding AT, heparin also binds other blood proteins, including platelet factor 4 (PF4 or CXCL4).[10,11] PF4 (released from alpha platelet granules) is a cationic chemokine and binds to the negatively charged heparin.[12] The interaction of heparin and PF4 is clinically significant, as it is associated with heparin-induced thrombocytopenia (HIT).[11]

Type II HIT is an adverse immunological effect of heparin therapy.[13-15] HIT occurs in a small percentage of patients (0.2% to 3%) undergoing heparin therapy and leading to a marked reduction in platelet count ($<1.5 \times 10^{11}$/L).[16] Untreated HIT can lead to life-threatening thrombosis, and clinical symptoms, including deep vein thrombosis, pulmonary embolism, cerebral sinus thrombosis,

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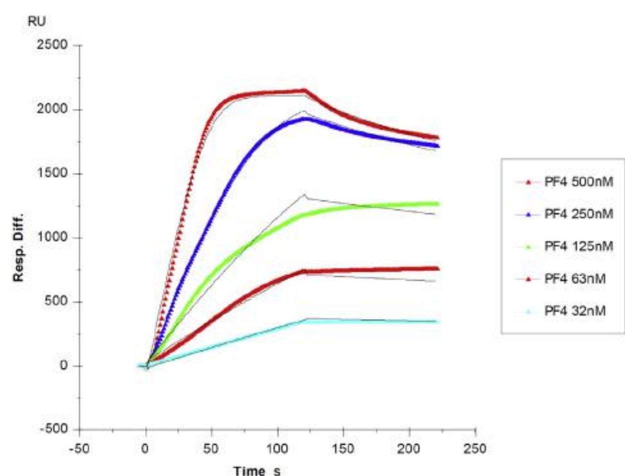

Figure 1. SPR sensorgrams of PF4−heparin interaction. Concentrations of PF4 (from top to bottom): 500, 250, 125, 62.5 and 32 nM, respectively. The PF4-heparin binding kinetics were determined by globally fitting the curves to a 1:1 biomolecular reaction model (black lines) using the BIAevaluation software package.

myocardial infarction, thrombotic stroke, lower limb arterial thrombosis, and skin necrosis. The onset of HIT occurs when heparin binds stoichiometrically to PF4 (e.g., 27 IU of heparin per milligram of PF4) and results in the formation of heparin−PF4 neoepitope.[14,16] Patients who have been previously exposed and sensitized to the heparin-PF4 immune complex produce anti−PF4−heparin antibodies. The major anti−PF4−heparin antibodies are immunoglobulin G, (IgG, 80%), and can include immunoglobulin A (IgA) or immunoglobulin M (IgM).[14,17,18] These anti−PF4−heparin antibodies (e.g., IgG) recognize and tightly bind to the heparin-PF4 immune complex, to form IgG-heparin-PF4 immune complex clusters (K_D, ~ 7-30 nM).[19] The Fc region of the antibody of this immune complex cluster recognizes and binds to FcγRIIa (CD32a) that is present on the platelet surface.[14,20,21] This interaction results in platelet activation and aggregation.[14,20,21] Activated platelets release procoagulants microparticles and serotonin.[22] These procoagulant microparticles increase thrombin generation leading to thrombosis. Serotonin release is a direct measure of heparin-dependent platelet activation, and currently, the presence of anti−PF4−heparin antibodies in a patients' serum can be evaluated using a serotonin release functional assay.[22] HIT induced platelet aggregation results in a decrease in platelet count ($<1.5 \times 10^{11}$/L or <50% of baseline platelet count).[16] Anti−PF4−heparin antibodies can also bind to heparan sulfate present on the endothelium leading to activation of tissue factor, and a cascading reaction resulting in endothelial damage.[14] The activation of tissue factor leads to increased production of thrombin, and platelet activation also results in an increase in PF4 release and subsequently aggravating the symptoms of HIT.[14]

Methods addressing the interaction of heparin and PF4 are essential for comparative studies (e.g., evaluation of heparins from different sources, lot variability, and process development of biosynthetic heparins and LMWH for NDAs and ANDAs).[23] The methods addressing biomolecular interaction of heparin and PF4 include: (1) PF4-heparin-binding kinetics (e.g., surface plasmon resonance method), (2) PF4-heparin complex size (e.g., turbidity assays, electron microscopy), and (3) complex charge (e.g., zeta potential method).[24] In addition, functional assays and *in vivo* assays are required to address the biological importance of HIT complex.[23] Current available HIT analytical methods that are mainly aimed at addressing the clinical management of HIT.[25] These methods evaluate the presence of anti-PF4-heparin antibodies in patient's blood

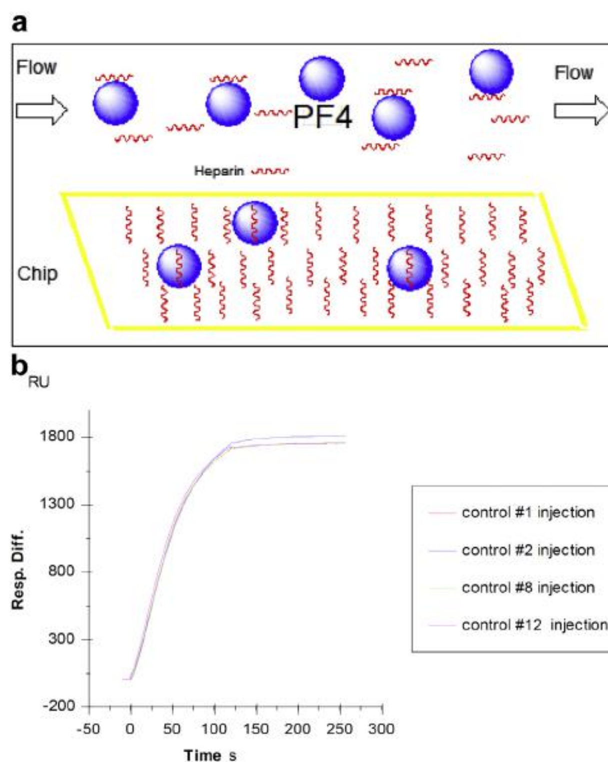

Figure 2. (a) The diagram of solution competition SPR assay; (b) Repeatable SPR sensorgrams of PF4-heparin interaction with different injection/binding and regenration times.

and whether these antibodies could activate platelet in the presence of heparin (e.g., serotonin release assay, platelet aggregation, expression of platelet membrane glycoproteins, activation of platelets, and generation of platelet-derived microparticles).[25]

The current study focuses on evaluating the binding affinity of heparin products to PF4, using a competitive SPR method. Surface plasmon resonance (SPR) is a real-time quantitative method for the analysis of biomolecular interactions, including binding affinity, specificity, and kinetics.[26-33] The current study focuses on developing a screening platform to study the interaction of heparin (ligand) and PF4 (analyte) by using an immobilized biotinylated heparin (ligand) on the biosensor chip. In addition, this heparin biosensor chip could be used to study the interaction of heparin and other heparin-binding proteins ubiquitously found in blood, thus serving as a screening tool to assess the immunogenicity and biological interactions of blood proteins and various heparin products.

Materials and Methods

Materials

Human PF4 was obtained from Hyphen BioMed (Neuville-sur-Oise, France). Porcine intestinal mucosa unfractionated heparin API samples were purchased from 4 different manufacturers; USP heparin reference, Celsus (Cincinnati, OH), Scientific Protein Laboratories (SPL, Waunakee, WI) and Tecoland (Irvine, CA). LMWH APIs from 4 different manufacturers, Sandoz, Aventis, Medefil, and Gland Pharma, were generously provided by Dr. Jawed Fareed of Loyala University Medical Center (Maywood, IL). Arixtra®, an ultra-LMWH, was obtained from Mylan Institutional LLC (Rockford, IL).

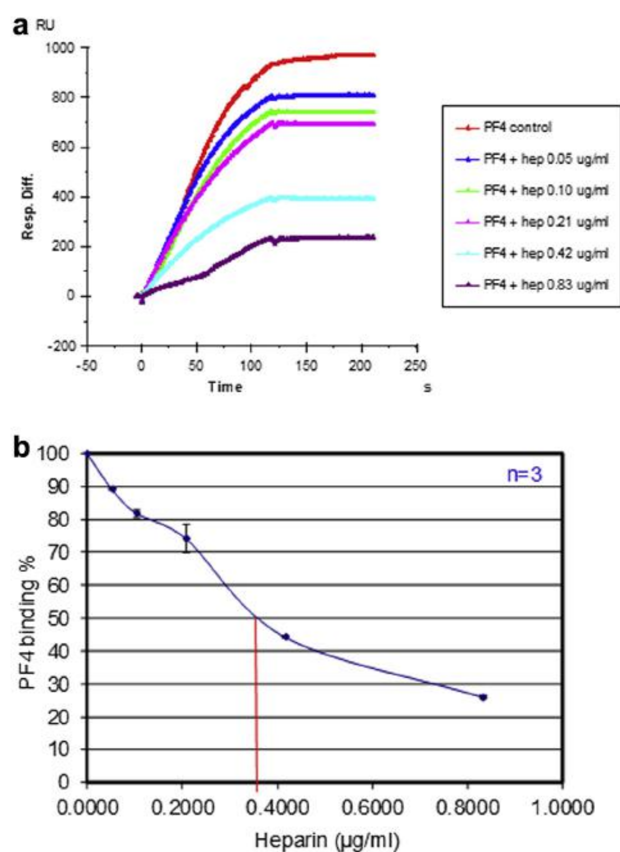

Figure 3. (a) Competition SPR sensorgrams of PF4—heparin interaction inhibiting by different concentration of heparin. PF4 concentration was 125 nM; (b) IC_{50} calculation of heparin using PF4 inhibition data from surface competition SPR.

SPR measurements were performed on a BIAcore 3000 (GE Healthcare, Uppsala, Sweden) operated using the manufacturer's software. Sensor SA Chips were from GE Healthcare.

Preparation of Heparin Biochip

Biotinylated heparin was prepared by using our previous protocol with minor modification.[32] Unfractionated heparin (2 mg) and 2 mg of amine—PEG_3—Biotin (Thermo Scientific, Waltham, MA) were mixed with 10 mg of $NaCNBH_3$ in 200 μL of H_2O for initial reaction, which was performed at 70°C for 24 h, and then a further 10 mg of $NaCNBH_3$ was added to continue the reaction for another 24 h. After completing this reaction, the mixture was desalted with a spin column (3000 molecular weight cut-off). The biotinylated heparin was immobilized to a streptavidin (SA)-coated chip based on the manufacturer's protocol. The successful immobilization of heparin was confirmed by the observation of a ~100 resonance unit (RU) increase on the sensor chip. The control flow cell (FC1) was prepared by 1 min injection with saturated biotin.

Kinetic Measurement of Interaction Between Heparin and PF4 Using Heparin Chip

The PF4 sample was diluted in HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4) (GE Healthcare, Uppsala, Sweden). Different dilutions of PF4 (from 10 to 500 nM) samples were injected at a flow rate of 40 μL/min. At the end of the sample injection, the same buffer was allowed to flow over the sensor surface to facilitate dissociation. After a 2 min dissociation time, the sensor surface was regenerated by sequential injecting with 40 μL of 10 mM glycin-HCl pH 2.5 buffer and 2 M NaCl to obtain fully regenerated surface. The response was monitored as a function of time (sensorgram) at 25°C.

SPR Solution Competition IC_{50} Measurement of Heparin and LMWH Products

Solution competition studies between surface heparin and soluble different heparin and LMWH to measure IC_{50} were performed using SPR.[30] In brief, PF4 (125 nM) samples alone or mixed with different concentrations of heparin, LMWH or ultra-LMWH in HBS-EP buffer were injected over the heparin chip at a flow rate of 40 μL/min, respectively. After each run, dissociation and regeneration were performed as described above. For each set of competition experiments, a control experiment (only protein without heparin or LMWH) was performed to ensure the surface was completely regenerated and that the results obtained between runs were comparable.

Results and Discussion

Kinetics Measurement of PF4-Heparin Interactions

Sensorgrams of the interaction of heparin with PF4 are shown in Figure 1. The binding sensorgrams fit reasonable well to a 1:1 Langmuir biomolecular reaction model. The interaction of PF4 with heparin displays an on-rate (k_{on}) of 2.3×10^5 ($\pm 7.1 \times 10^3$) M^{-1} s^{-1} and an off-rate (k_{off}) of 2.6×10^{-3} ($\pm 1.0 \times 10^{-4}$) s^{-1} (±standard errors were obtained from the global fitting of injections at 5 different concentrations). The binding equilibrium dissociation constant ($K_D = k_{off}/k_{on}$) for the PF4—heparin interaction was determined to be 11 nM.

SPR Solution Competition IC_{50} Measurement of Heparin Products

The SPR method for solution competition between surface heparin (immobilized on the chip surface) and soluble different heparin was developed. We optimized the regeneration conditions for the sensor surface by sequentially injecting 40 μL of 10 mM glycine-HCl pH 2.5 buffer and 2 M NaCl. The heparin surface was fully regenerated and the heparin-containing chip was stable for >100 PF4-heparin interaction runs (Fig. 2b).

SPR Solution Competition IC_{50} Measurement of Heparins and LMWHs

Solution competition between surface immobilized heparin and various soluble heparins was performed to measure IC_{50} values (Figs. 3a and 3b). PF4 (125 nM) samples were pre-mixed with different

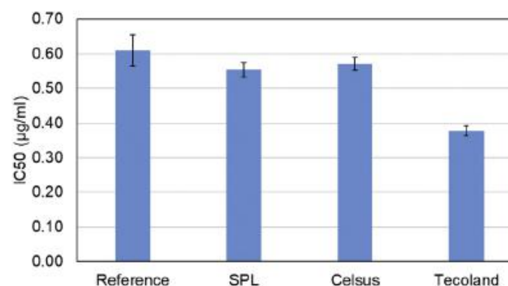

Figure 4. IC_{50} values (μg/mL, based on quadruplicate experiments) of heparin (API) from different manufacturers on inhibiting PF4 binding to heparin (on chip surface).

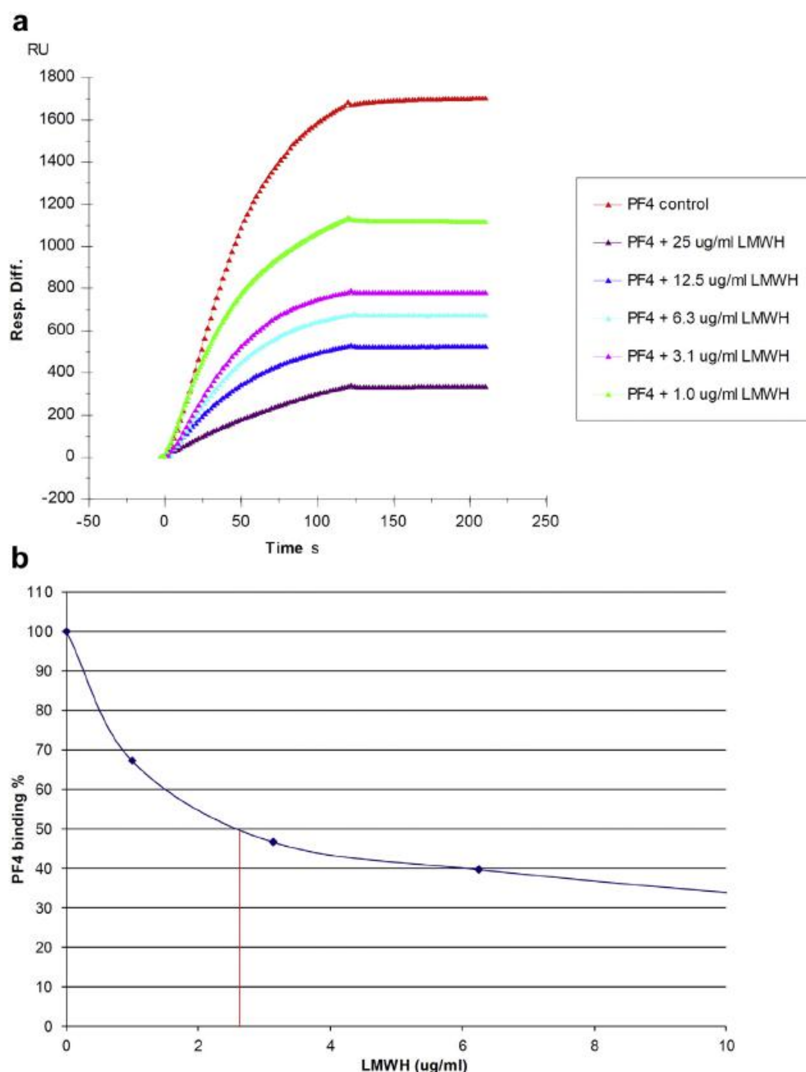

Figure 5. (a) Competition SPR sensorgrams of PF4–heparin interaction inhibiting by different concentration of LMWH. PF4 concentration was 125 nM; (b) IC_{50} calculation of heparin using PF4 inhibition data from surface competition SPR.

concentrations of heparin before injection to the heparin chip. The sensorgrams (Fig. 3a) show that once the active binding sites on PF4 were occupied by heparin in solution, the binding of PF4 to the surface-immobilized heparin decreased resulting in a reduction of signal in a concentration dependent fashion. The IC_{50} values concentration of competing analyte resulting in a 50% decrease in response units (RU) can be calculated from the plots PF4 binding signal (normalized) versus heparin or LMWH concentration in solution (Fig. 3b).

A summary of IC_{50} of heparins from different manufacturers on inhibiting PF4 binding to surface heparin is shown in Figure 4. The IC_{50} values of different heparins varied in the range from 0.38 to 0.6 μg/mL, suggesting the binding affinity of heparins to PF4 is different. The lower IC_{50} of Tecoland heparin could be due to a combinatorial effect of differences in MW, the net negative charge/sulfation pattern of the heparin chain or the possible presence of impurities in the heparin. Future experiments aim to address factors that may affect PF4-heparin-binding, and include, the effect of MW of heparin, the net charge of the heparin chain, sulfation patterns and process impurities.

Similarly, solution competition between surface-bound heparin and soluble LMWHs was performed to measure IC_{50} for LMWHs (Figs. 5a and 5b). A summary of LMWH IC_{50} values from different LMWH APIs on inhibiting PF4 binding to heparin is shown in Figure 6. The IC_{50} values of different LMWHs are comparable, ranging from 2.4 to 2.9 μg/mL, which are much higher than the IC_{50} values of heparin, demonstrating that the binding affinity of

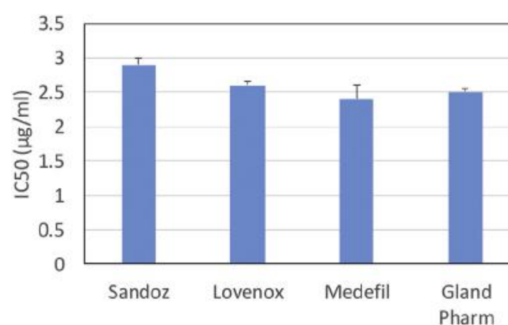

Figure 6. IC_{50} values (μg/mL, based on quadruplicate experiments) of LMWHs from different manufacturers on inhibiting PF4 binding to heparin (on chip surface).

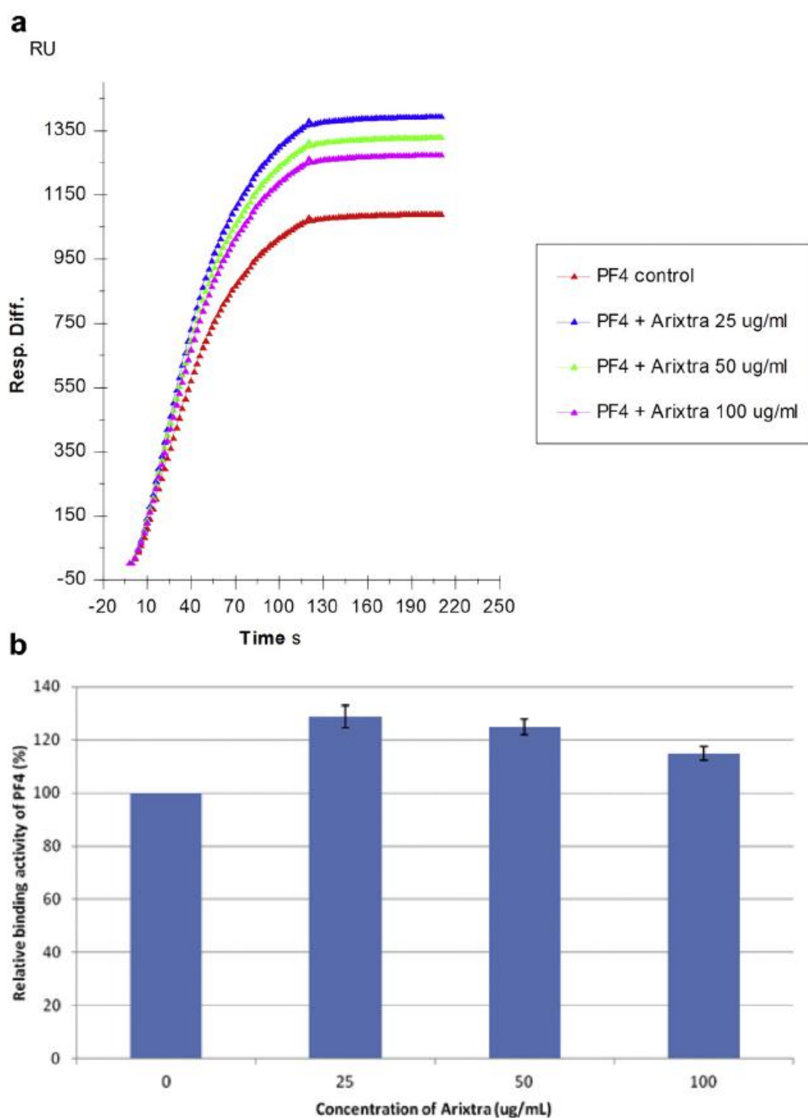

Figure 7. (a) SPR sensorgrams of PF4—heparin interaction by adding with different concentration of Arixtra®. PF4 concentration was 125 nM, Concentrations of Arixtra®: 0, 25, 50 and 100 μg/mL. (b) Bar graphs (based on triplicate experiments) illustrating the effect of Arixtra® on the PF4 binding to immobilized heparin on the chip surface.

heparins to PF4 is much greater than LMWH, and thereby, resulting in a higher potential of HIT for unfractionated heparin. The IC_{50} values obtained for LMWHs can be used to calculate apparent dissociation constants (K_{Dapp}) in the range of 43 to 52 nM for the LMWH—PF4 interaction by using Equation 1:

$$K_{Dapp} = IC_{50}/(1 + [C]/K_D) \quad (1)$$

where [C] is the concentration of PF4 used in the IC_{50} measurement, and K_D is the binding equilibrium dissociation constant for the PF4—heparin interaction.

SPR Solution Competition With Arixtra®

Arixtra® is a synthetic pentasaccharide factor Xa inhibitor, which binds AT and accelerates its inhibition of factor Xa.[1] PF4 (125 nM) samples mixed with different concentrations (from 25 to 100 μg/mL) of Arixtra® were injected to the heparin surface. As shown in Figures 7a and 7b, no apparent inhibition was detected despite use of very high concentrations of Arixtra®. This suggests that the potential of HIT for Arixtra® is very low.

Conclusion

We developed a rapid method for the evaluation of the HIT potential of heparin products using solution competition SPR. Solution competition between surface-immobilized heparin and soluble heparin products, including unfractionated heparin, LMWH or ultra-LMWH (Arixtra®), that bind to PF4 was performed leading to IC_{50} determination for different heparin products. The IC_{50} values of different unfractionated heparin APIs varied from 0.38 to 0.6 μg/mL and the IC_{50} values of different LMWH APIs ranged from 2.4 to 2.9 μg/mL. The IC_{50} of Arixtra® was not measurable even at very high concentrations. These IC_{50} values for different heparin products provide a rapid, reproducible and quantitative evaluation of the HIT potential of various heparin products.

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