



Development of a fluorescent anti-factor Xa assay to monitor unfractionated and low molecular weight heparins

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ABSTRACT

Fluorogenic assays have many potential advantages over traditional clot-based and chromogenic assays such as the absence of interference from a range of factor deficiencies as well as offering the possibility of assays in platelet rich plasma or whole blood. A fluorogenic anti-factor Xa (anti-FXa) assay has been developed for the determination of unfractionated heparin (UFH), low molecular weight heparins (LMWHs), namely enoxaparin and tinzaparin, and the synthetic heparinoid danaparoid, in commercial human pooled plasma. The assay was based on the complexation of heparin-spiked plasmas with exogenous FXa at a concentration of 4 nM in the presence of 0.9 μM of the fluorogenic substrate methylsulfonyl-D-cyclohexylalanyl-glycyl-arginine-7-amino-4-methylcoumarin acetate (Pefalfluor FXa). Pooled plasma samples were spiked with concentrations of anticoagulants in the range 0–1.6 U/ml. The assay was capable of the measurement of UFH and danaparoid in the range 0–1 U/ml, and enoxaparin and tinzaparin in the range 0–0.8 and 0–0.6 U/ml, respectively. Correlation coefficients generated by linear regression of the log/lin data analysis were between 0.93 and 0.96 for the anticoagulants tested. Assay percentage coefficients of variation were typically below 7%.

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1. Introduction

Anticoagulant drugs are routinely used for a wide range of clinical indications, including the prevention and treatment of venous thromboembolism (VTE) and the acute management of ischaemic heart disease [1,2]. Unfractionated heparin (UFH) constitutes the most widely used parenteral anticoagulant therapy [3,4], and consists of a heterogeneous mixture of negatively charged glycosaminoglycans derived from either bovine lung, or porcine intestine. UFH exerts its anticoagulant effect primarily through binding to plasma antithrombin (AT) [5]. This binding causes a conformational change in antithrombin, which markedly enhances the ability of antithrombin to specifically inhibit procoagulant factors Xa (FXa) and thrombin (IIa) [1].

Low molecular weight heparins (LMWHs) are prepared by chemical or enzymatic depolymerisation of heterogeneous UFH glycosaminoglycan chains [1,6]. LMWHs demonstrate better bioavailability, have significantly longer half-lives compared to UFH [1], and are also associated with reduced incidence of significant complications [7]. Consequently, UFH has been replaced by

LMWH as the treatment of choice for many indications. Although the LMWHs share many pharmacodynamic properties, commercial products differ significantly with respect to final molecular weights (4000–7000 Da) [4]. Although the LMWHs all bind to AT, they demonstrate different abilities to enhance inactivation of FXa and/or thrombin (anti-FXa:anti-FIIa ratios ranging from 2:1 to 4:1) [8]. Danaparoid is prepared from bovine and porcine mucosa, and is classed as a low molecular weight heparinoid which has an anti-FXa:anti-IIa ratio of $\geq 22:1$ [9].

Despite its widespread use, it is well recognized that UFH has a relatively narrow therapeutic window [1]. Consequently, laboratory monitoring and dose-titration of UFH therapy is a standard clinical practice. In contrast, because of more predictable pharmacokinetic profiles, LMWH therapy typically requires less laboratory monitoring. A number of different laboratory assays have been used to enable adjustment of heparin doses. Most commonly used are clot-based assays in the form of the activated partial thromboplastin time (APTT) [10] and the activated clotting time (ACT) [11]. Nevertheless, it is well established that these assays have a number of important limitations. First, since the APTT is a standard end-point clotting time assay, it can be influenced by many other coagulation variables. For example, the APTT will be prolonged by inherited or acquired coagulation factor deficiencies (including factors XII, XI, X, IX, VIII, V, and II) [8,12]. Second, more than

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300 different laboratory tests are in clinical use to measure the APTT. Previous reports have clearly shown that varying combinations of different commercial reagents and coagulometer machines can result in marked inter-laboratory variability in responsiveness to therapeutic UFH concentrations [13,14]. Similarly, although the ACT is widely used to monitor heparin reversal in patients during cardiac surgery, it is also associated with important limitations [15].

Since the APTT is generally insensitive to LMWH, plasma anti-FXa activity monitoring is established as the assay of choice for those patients who require monitoring. In addition, the anti-FXa assay can also be used to monitor UFH levels. Moreover, since the anti-FXa assay generally involves a chromogenic end-point, use of this assay to monitor UFH levels is attractive in that it is not affected by many of the other biologic variables that can interfere with clot-based end-points. While chromogenic assays confer many advantages over standard clot-based assays, there are some drawbacks. The use of chromogenic substrates requires measuring optical density, which renders whole blood and platelet rich plasma samples problematic for colorimetric measurements [16]. Fibrinogen clotting results in turbidity of plasma samples which interferes negatively with absorbance readings [16–18]. Also the lack of a standard anti-FXa chromogenic assay gives rise to significant inter-variability between commercially available assays [13,19].

The potential advantages of fluorogenic assays over chromogenic assays include the ability to use a range of sample types such as platelet poor (PPP), platelet rich (PRP) and whole blood samples, as fluorescence is not as influenced by the opacity of the sample as absorbance [18]. Although fluorogenic assays to assess thrombin generation have been developed, there is accumulating evidence that FXa may represent a better target as it occupies a critical junction in the coagulation cascade [20]. In this study, we have sought to develop a novel plate-based fluorogenic anti-FXa assay that is sensitive to pharmacological concentrations of UFH, LMWHs, and danaparoid.

2. Materials and methods

2.1. Reagents

Water (ACS reagent), HEPES (minimum 99.5% titration) and sodium citrate tribasic dehydrate (ACS reagent, 99.0%) were all from Sigma–Aldrich (Dublin, Ireland). Filtered HEPES and sodium citrate solutions had concentrations of 10 mM (pH 7.4) and 0.1 M (pH 5.5), respectively. A 100 mM filtered stock solution of CaCl_2 from Fluka BioChemika (Buchs, Switzerland) was prepared from a 1 M CaCl_2 solution. The fluorogenic substrate methylsulfonyl-D-cyclohexylalanyl-glycyl-arginine-7-amino-4-methylcoumarin acetate (Pefalfluor FXa) was purchased from Pentapharm (Basel, Switzerland). It was reconstituted in 1 ml of water having a final concentration of 10 mM, aliquoted and stored at -20°C until further use. Dilutions from 10 mM stock solutions down to 10 μM were freshly prepared with water when needed. Subsequent dilutions were prepared in 10 mM HEPES. Tubes were covered with aluminum foil to protect from exposure to light. Purified human Factor Xa (FXa) was obtained from HYPHEN BioMed (Neuville-Sur-Oise, France). Tinzaparin (Innohep[®]) was obtained from LEO Pharma (Ballerup, Denmark). UFH, enoxaparin (Clexane[®]) and danaparoid (Orgaran[®]) were from Sigma–Aldrich (Missouri, USA), Sanofi–Aventis (Paris, France) and Schering–Plough (New Jersey, USA), respectively. Human pooled plasma was purchased from Helena Biosciences Europe (Tyne and Wear, UK). Lyophilised plasma was reconstituted in 1 ml of water and left to stabilize for at least 20 min at room temperature prior to use.

2.2. Apparatus and software

Fluorescence intensities were measured in a Spectrophotometer Infinite M200 microplate reader from Tecan Group Ltd. (Männedorf, Switzerland) equipped with a UV Xenon flashlamp. Flat, black-bottom 96-well polystyrol FluorNunc[™] microplates from Thermo Fisher Scientific (Roskilde, Denmark) were used.

2.3. Fluorogenic anti-FXa assay

Measurements were carried out in reconstituted citrated human pooled plasma without the addition of exogenous AT. FXa and Pefalfluor FXa were titrated within the range of 0.1–10 nM and 0.1–100 μM ($K_m = 220 \mu\text{M}$), respectively. Each well contained 6.25 μl of 100 mM CaCl_2 , 43.75 μl of pooled plasma, and 50 μl of FXa (0.1–10 nM). The reaction was started by adding 50 μl of Pefalfluor FXa fluorogenic substrate (0.1–100 μM). Samples within wells were mixed with the aid of orbital shaking at 37°C for 30 s. Immediately after shaking, fluorescence measurements were recorded at 37°C for 60 min, at 20 μs intervals. Fluorescence excitation was at 342 nm and emission was monitored at 440 nm, corresponding to the excitation/emission wavelengths of the 7-amino-4-methylcoumarin (AMC) fluorophore. All the measurements were carried out in triplicate. Following optimization of assay conditions, pooled commercial plasma samples were spiked with pharmacologically relevant concentrations (0–1.6 U/ml) of therapeutic anticoagulants including UFH, enoxaparin, tinzaparin and danaparoid. In all experiments, reaction progress curves were obtained and analyzed in SigmaPlot 8.0. The reaction rate (slope), which is defined as the change in fluorescence divided by the change in time (i.e. dF/dt), was measured as the linear portion of the fluorescence response profile and plotted versus all different anticoagulant concentrations.

2.4. Statistical analysis

Inter- and intra-assay differences between anticoagulant concentrations were compared using one-way analysis of variance (ANOVA), with subsequent post hoc analysis performed (Scheffe test) if significance was observed. A result of $p < 0.05$ was considered statistically significant. SPSS 15.0 was used for data processing and analysis.

3. Results

3.1. Assay optimization

In standard anti-FXa assays, excess FXa added to heparinized plasma is inhibited by the heparin–AT complex, and residual FXa activity is assessed using a chromogenic substrate [19,21]. Our assay measures the rate of AMC fluorophore released by FXa substrate cleavage, which is inversely dependent upon plasma heparin(oid) concentration. To establish optimal assay concentrations, Pefalfluor FXa fluorogenic substrate and FXa were titrated over a range of concentrations. Pefalfluor FXa substrate concentration was evaluated within the range of 0.1 μM (0.45×10^{-3} times K_m) to 100 μM (0.45 times K_m) with FXa in excess of physiological concentrations (150 pM) [22] at a concentration of 1 nM. Pefalfluor FXa concentrations were chosen on the basis of working below and around the K_m value to allow differentiation in reaction rates at different anticoagulant concentrations, as the difference in reaction rates at substrate concentrations larger than the K_m is almost negligible. An example of the fluorogenic assay FXa/substrate titration (0.8 μM substrate and 0.1, 1 and 10 nM FXa concentrations) can be seen in Fig. 1. The optimized assay is capable of differentiating the concentration of UFH in commercial human pooled plasma

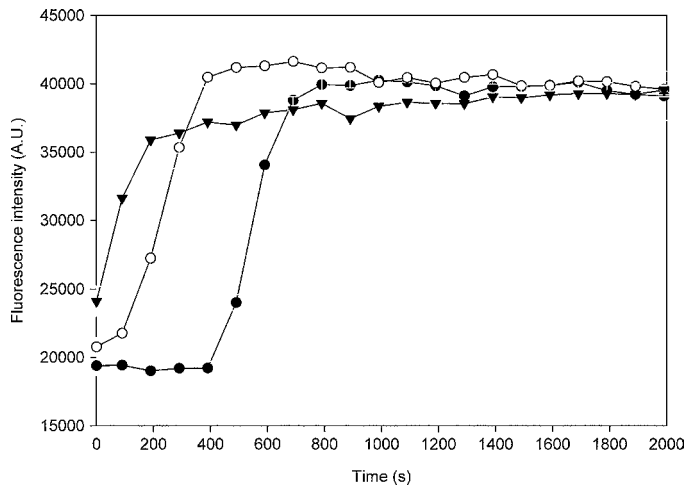


Fig. 1. Fluorescence intensity vs. time for the fluorogenic assay consisting of $0.8 \mu\text{M}$ Pefalfluor FXa and 2 mM CaCl_2 in human pooled commercial plasma in the presence of 0.1 nM (\bullet), 1 nM (\circ), and 10 nM (\blacktriangledown) FXa concentrations.

from 0 to 1 U/ml , at a final assay concentration of 4 nM FXa and $0.9 \mu\text{M}$ Pefalfluor FXa.

3.2. Evaluation of the anti-FXa fluorogenic assay for the quantitative determination of anticoagulant dose–response and assay variability

Four heparin drugs were tested using the optimized fluorogenic anti-FXa assay in commercial human pooled plasma. The reaction progress curves were similar for all four drugs in that, as anticoagulant concentration increased, lag times were extended and reaction rates were reduced. This is illustrated in Fig. 2 (inset) which shows the fluorescence profiles of the anti-FXa assay response to UFH. For each type of anticoagulant, the reaction curves reached a plateau at approximately the same level ($48,000$ – $53,000 \text{ AU}$) independent of anticoagulant concentration. This upper response limit was dictated by the substrate (and thus product) concentration and the upper detection limit of the instrument.

The dose–response profile for the assay in UFH was calculated using the linear regions of the fluorescence responses. It can be observed that the assay was capable of the differentiation of UFH concentrations from 0.2 to 1.6 U/ml at intervals of 0.2 U/ml . It

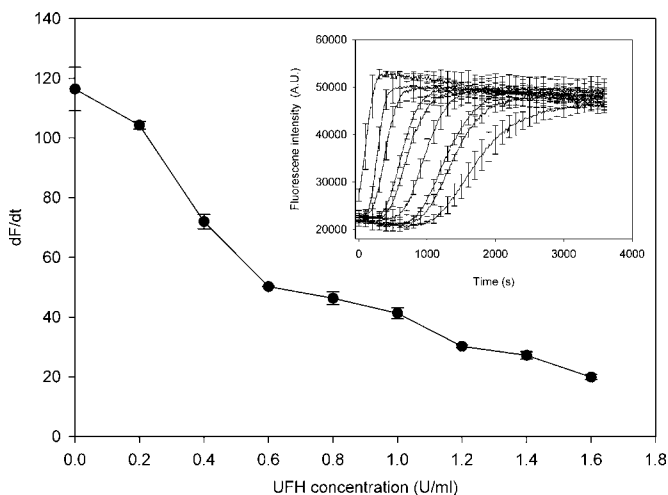


Fig. 2. Dose–response curve of UFH in pooled plasma on fluorogenic anti-FXa assay ($n = 3$), calculated from the linear slopes of the fluorescence response curves. Inset: Fluorescence intensity vs. time for the anti-FXa activity with UFH ($n = 3$).

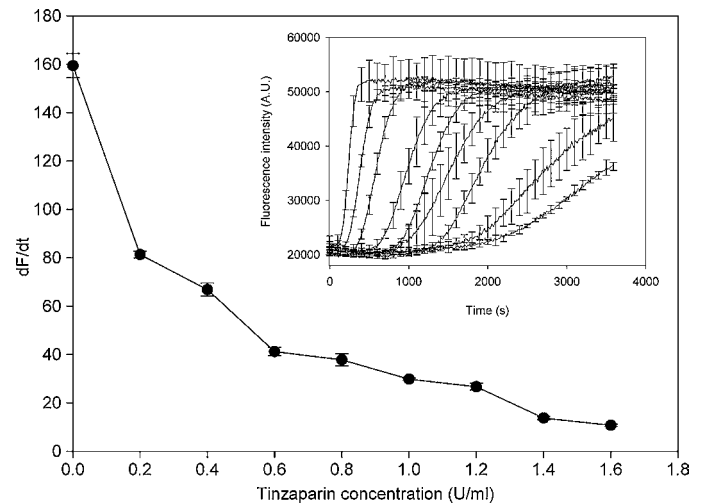


Fig. 3. Dose–response curve of tinzaparin in pooled plasma on fluorogenic anti-FXa assay ($n = 3$), calculated from the linear slopes of the fluorescence response curves. Inset: Fluorescence intensity vs. time for the anti-FXa activity with tinzaparin ($n = 3$).

has previously been reported that non-linearity is observed with wide-ranging concentrations of heparin [23] and that the half-life and intensity of the effects of heparin rise disproportionately with increasing heparin dose [4]. Highest assay sensitivity can be observed at lower heparin concentrations (0 – 0.6 U/ml) but at the upper range (0.6 – 1.6 U/ml) the sensitivity of the assay decreased significantly. Response slopes to UFH concentrations at 0.2 U/ml intervals were statistically different from one another ($p < 0.001$) up to 1 U/ml UFH except for UFH concentrations of 0.6 and 0.8 U/ml between which there was no statistical difference ($p = 0.441$).

The anti-FXa assay was tested further on a range of anticoagulant drugs, namely tinzaparin, enoxaparin and danaparoid. The fluorescence response profiles of the fluorogenic anti-FXa assay to tinzaparin and the associated dose–response curve can be seen in Fig. 3. Typically, the responses reached a plateau of around $50,000$ – $55,000 \text{ AU}$ and lag times increased while the slopes of the linear response region decreased with increasing concentration. In nearly all instances there were statistically significant differences between the slopes of the adjacent drug concentrations ($p < 0.001$). The resulting dose–response curve showed higher sensitivity in the lower tinzaparin range (0.2 – 0.6 U/ml). At higher concentrations of the anticoagulant, assay sensitivity again decreased, which was similar to that observed for UFH. However, the highest dose tested (1.6 U/ml) could still be distinguished from the next concentration of 1.4 U/ml ($p < 0.001$). LMWHs have been reported as giving a more predictable dose–response than UFH due to better bioavailability at low doses.

The effect of enoxaparin was also evaluated using the fluorogenic anti-FXa assay (Fig. 4). Fluorescence response curves showed comparable characteristics to UFH and tinzaparin and again reached a plateau around $50,000 \text{ AU}$. However, the dose–response curve shows that, although the anti-FXa assay had good sensitivity to enoxaparin between 0 and 0.4 U/ml and retained moderate sensitivity up to 0.8 U/ml , at concentrations greater than 1.0 U/ml the assay showed a significant loss in sensitivity ($p > 0.05$).

Fig. 5 shows the fluorescence responses from the anti-FXa assay in the presence of danaparoid. Again, similar to earlier results, the fluorescence responses reached a plateau between $45,000$ and $50,000 \text{ AU}$. However, the lag times were more prolonged in the presence of danaparoid than in the presence of UFH. For example, at 0.8 U/ml danaparoid, the lag time ended at 2000 s compared to UFH which ended at 500 s . The slopes in the presence of danaparoid were also much lower than for UFH. This is likely due to

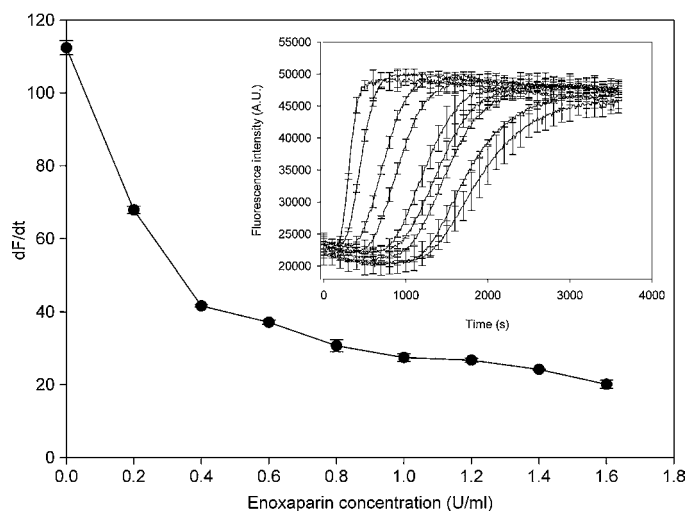


Fig. 4. Dose–response curve of enoxaparin in pooled plasma on fluorogenic anti-FXA assay ($n = 3$), calculated from the linear slopes of the fluorescence response curves. Inset: Fluorescence intensity vs. time for the anti-FXA activity with enoxaparin ($n = 3$).

the more complete inhibition of FXa by danaparoid as a result of its high anti-FXa:anti-FIIa activity ratio of $\geq 22:1$ [9]. At 1.2 U/ml the reproducibility also became a significant issue with a %CV of 21%. Above this, it was not possible to calculate meaningful slopes. This assay was found to be statistically sensitive to danaparoid up to concentrations of 1 U/ml ($p < 0.001$). While danaparoid has little effect on standard clotting tests [4] it can be monitored with the anti-FXA fluorogenic assay developed in this study quite reliably.

Also critical to assay performance is the relative standard deviation or percentage coefficient of variation (%CV). Table 1 summarizes the analytical errors associated with each of the slope measurements, namely the standard deviation and the percentage coefficient of variation for each anticoagulant drug in the anti-FXA assay. CV values for all drugs tested were $< 7\%$ for the plate-based assay (except for a single point at 1.2 U/ml danaparoid) which are in line with commercially available assays [24,25] and the WHO Expert Committee on Biological Standardization report [26].

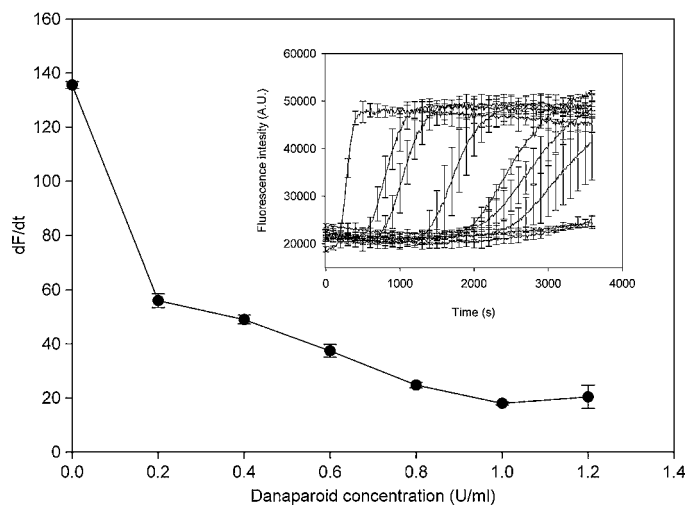


Fig. 5. Dose–response curve of danaparoid in pooled plasma on fluorogenic anti-FXA assay ($n = 3$), calculated from the linear slopes of the fluorescence response curves. Inset: Fluorescence intensity vs. time for the anti-FXA activity with danaparoid ($n = 3$).

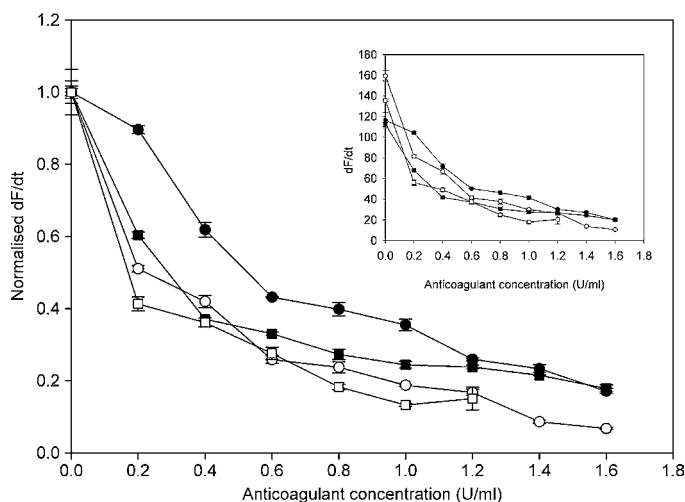


Fig. 6. Dose–response curves (normalized data) of the fluorogenic anti-FXA assay to UFH (●), tinzaparin (○), enoxaparin (■), and danaparoid (□) in commercial human pooled plasma ($n = 3$). Inset: Dose–response curves (raw data) of the fluorogenic anti-FXA assay to UFH (●), tinzaparin (○), enoxaparin (■), and danaparoid (□) in commercial human pooled plasma ($n = 3$).

3.3. Comparison of responses of the anti-FXA assay to different drugs

The inter-assay variability between drugs was also assessed. While the general trend for each drug was similar (Fig. 6), specific differences were observed in the response of each drug. An indication of the inter-assay variation is given by the results for the 0 U/ml measurements for the four assays. As can be seen in Fig. 6, this value varied from approximately 115 to 160 AU/s. This variation could be due to variations in antithrombin activity levels in the control plasma, variations in exogenous Xa activity levels, or other experimental variables such as time and temperature. As a result, the inter-assay variability was 16%.

Statistical analysis of the raw data indicated that concentrations of 0.2, 0.4, and 0.8 U/ml, for all four drugs tested were significantly different from one another ($p < 0.001$) with the exception of tinzaparin and UFH at 0.4 U/ml ($p = 0.78$). At 0.6 and 1 U/ml the Levene's test was $p = 0.020$ and $p = 0.032$, respectively. Hence, there were significant differences in the variances and therefore one-way ANOVA could not be applied. At concentrations of 1.2, 1.4, and 1.6 U/ml, statistical analysis showed significant differences between UFH, enoxaparin, and tinzaparin. Danaparoid data was not recorded within this range.

To eliminate differences due to inter-assay variation, ratios of the dose–responses were made against those at 0 U/ml heparin. Statistical analysis of this normalized data showed specific differences from the raw data. UFH was seen to be distinctive from the LMWHs and danaparoid where it showed comparatively less decrease in assay activity for comparable doses, particularly at lower concentrations. Concentrations of 0.2, 0.8, 1, 1.4 and 1.6 U/ml for all four drugs tested were significantly different ($p < 0.001$). However at 0.8 U/ml, tinzaparin and enoxaparin were not significantly different ($p = 0.069$). Also, UFH and enoxaparin were not significantly different at 1.4 U/ml ($p = 0.064$) and 1.6 U/ml ($p = 0.433$). At concentrations of 0.4 U/ml ($p = 0.043$), 0.6 U/ml ($p = 0.011$) and 1.2 U/ml ($p = 0.004$) significant differences were identified in the variances, so one-way ANOVA could not be applied.

The responses of all four drugs to the anti-FXA fluorogenic assay can be further compared using the reaction rates of the normalized data. UFH at 0.2, 0.4, 0.8, 1.2, 1.4 and 1.6 U/ml returned higher reaction rates than both LMWHs and danaparoid. Enoxaparin reaction rates were higher than tinzaparin reaction

Table 1
Percentage coefficient of variation (%CV) and standard deviation (SD) for all anticoagulants tested ($n = 3$).

Concentration (U/ml)	UFH		Tinzaparin		Enoxaparin		Danaparoid	
	SD	%CV	SD	%CV	SD	%CV	SD	%CV
0	7.29	6.26	5.03	3.16	1.94	1.73	1.3	0.96
0.2	1.29	1.23	1.43	1.75	1.03	1.52	2.55	4.56
0.4	2.42	3.36	2.68	4.00	0.30	0.73	1.64	3.35
0.6	0.17	0.33	1.70	4.13	0.56	0.50	2.34	6.27
0.8	2.13	4.61	2.48	6.55	1.62	5.28	1.02	4.13
1.0	1.87	4.54	0.42	1.39	10.5	3.83	0.54	3.01
1.2	0.42	1.4	1.40	5.25	0.57	2.12	4.27	20.95
1.4	1.25	4.59	0.71	5.14	0.18	0.74	–	–
1.6	0.83	4.19	0.44	4.13	1.11	5.52	–	–

rates at 0.2, 0.8, 1.4 and 1.6 U/ml but at all other concentrations the reaction rates were the same. Danaparoid returned the lowest reaction rates. This pattern could relate to the molecular weights of each drug with UFH at 11393 Da, tinzaparin at 5866 Da and enoxaparin at 4371 Da and danaparoid at a similar molecular weight to LMWHs. At higher molecular weights, greater reaction rates were calculated indicating greater sensitivity of the assay to that particular drug. In addition a trend can be observed between reaction rates and anti-FXa:anti-IIa ratios which also correlates with the pattern observed with molecular weight, e.g. UFH > tinzaparin > enoxaparin > danaparoid: 1:1 > 1.9:1 > 2.7:1 \geq 22:1. A high anti-FXa:anti-IIa ratio and low molecular weight translates into a lower reaction rate value.

Linear regression analysis was also used to compare all anticoagulant responses to the anti-FXa assay. Fig. 7 shows the log/lin plot of the dF/dt value of each anticoagulant against its concentration within its statistically sensitive range. The following correlation coefficients and slopes were calculated for each anticoagulant: $R^2 > 0.95$, slope = $-0.5 \log(dF/dt) \text{ ml U}^{-1}$ for UFH; $R^2 > 0.93$, slope = $-0.7 \log(dF/dt) \text{ ml U}^{-1}$ for tinzaparin; $R^2 > 0.96$, slope = $-0.9 \log(dF/dt) \text{ ml U}^{-1}$ for enoxaparin; $R^2 > 0.94$, slope = $-0.8 \log(dF/dt) \text{ ml U}^{-1}$ for danaparoid.

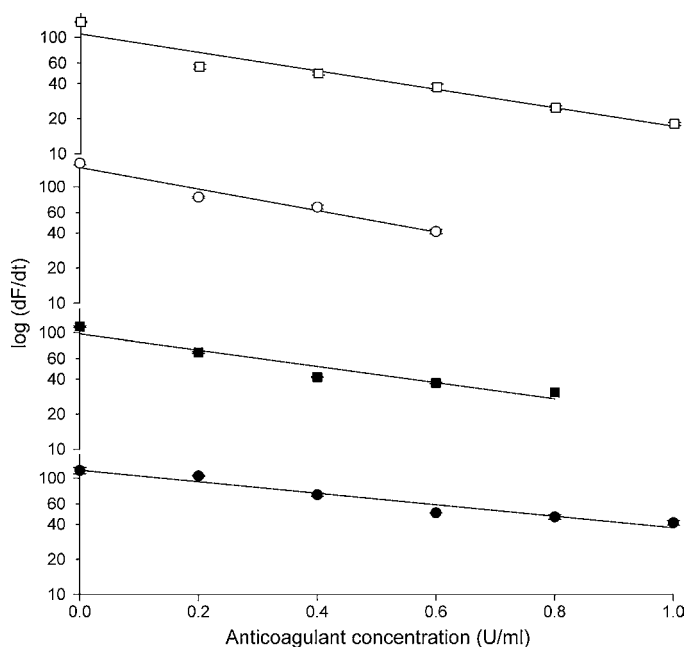


Fig. 7. Log/lin plot of dF/dt vs. concentration of UFH (●), tinzaparin (○), enoxaparin (■), and danaparoid (□) in commercial human pooled plasma ($n = 3$).

4. Discussion

Injectable drugs such as UFH, LMWHs and associated synthetic heparinoids are widely used and extremely efficacious anticoagulant treatments. However, UFH needs careful monitoring as it suffers significantly from a narrow therapeutic window and the subsequent harmful implications of clotting or bleeding. As already discussed, monitoring of UFH has long relied on clot-based assays (APTT and ACT) which have significant limitations. Furthermore, although LMWHs require less laboratory monitoring due to their predictable pharmacokinetics, monitoring is recommended in populations where pharmacokinetic parameters are altered, including obesity, renal insufficiency, pregnancy, in underweight patients, elderly patients, and children [7,27].

The aim of this study was to develop a novel, reproducible, and sensitive assay using fluorescence to quantify unfractionated heparin, enoxaparin, tinzaparin and danaparoid in human pooled plasma. Consequently, we measured the effect of these four anticoagulants on the fluorogenic anti-FXa assay at anticoagulant concentrations of 0–1.6 U/ml. For the purpose of assay development human pooled plasma was used. It is well known that autofluorescence is associated with human plasma due to the presence of many proteins such as tryptophan, bilirubin and others. The autofluorescence of these proteins varies between patients due to pH variation and the intrinsic characteristics of each individual [28]. Such variations in plasma background fluorescence can be corrected through the incorporation of appropriate controls including the use of background subtraction measurement techniques. The intra-assay variability and sensitivity limit was established for each of these four drugs. In addition we investigated the inter-variability of the assay for all drugs, so the assay for UFH was compared with the assay for LMWHs and danaparoid. SPSS statistical analysis proved that there were significant differences in assay sensitivity between drugs.

In the study presented here the anti-FXa assay for UFH was statistically differentiated at intervals of 0.2 up to 1 U/ml and showed excellent reproducibility with CVs below 7%. The anti-FXa assay was also statistically sensitive up to 0.6 and 0.8 U/ml for tinzaparin and enoxaparin, respectively. Assay reproducibility for both LMWHs was excellent with CVs of 0.5–7%. Linear regression analysis of the log/lin data resulted in correlation coefficients of 0.93–0.96 for all drugs.

The fluorogenic anti-FXa assay developed in this study compares favorably with other commercially available anti-FXa assays for central laboratory testing in terms of linearity and precision. The following are all chromogenic assays and measure therapeutic levels of both UFH and LMWH. The ACTICHRONE[®] Heparin (anti-FXa) assay from American Diagnostica Inc., can quantify up to 0.8 U/ml with reproducibility of 5–10%. The three assays available from Chromogenix, COACUTE[®], COATEST[®] and COAMATIC[®] detect levels of 0–1 U/ml. The intra- and inter-variability of these assays ranges from 1 to 6%. The HemosIL[™] Heparin kit from Instrumen-

tation Laboratory measures up to 1 U/ml with higher CVs of 9–12%. Hyphen BioMed supply the BIOPHEN[®] Factor X assay which can differentiate concentrations of up to 2 U/ml with CVs of less than 5%. Considering the performance characteristics of commercially available chromogenic kits, the fluorogenic anti-FXa assay developed in this study offers several advantages such as the ability to measure in a range of sample types including PPP, PRP and whole blood. Furthermore one unique assay has been developed that has the scope to monitor four different anticoagulants with reproducible assay precision and linearity in the statistically sensitive range.

The use of a point of care chromogenic anti-FXa assay for monitoring enoxaparin in patients who have undergone percutaneous coronary intervention (PCI) has also been described [29]. The chromogenic ENOX assay described can detect anti-FXa levels of 0.63–1.34 U/ml. However, at lower enoxaparin concentrations, the sensitivity of the assay decreases. An analytical range of 0.5–1 U/ml is appropriate for PCI [30,31] and the fluorogenic assay presented here covers this therapeutic range as well as low dose enoxaparin (0.2–0.8 U/ml).

The anti-FXa:anti-FIIa ratios for enoxaparin and tinzaparin are 2.7:1 and 1.9:1, both exerting a larger inhibitory effect on FXa than thrombin. As has already been stated, LMWHs have less of an effect on ACT and APTT than UFH [6], supporting the theory that prolongation of these tests is dependent upon inhibition of thrombin rather than FXa, highlighting the need for an anti-FXa activity test. The use of a single calibration curve for monitoring LMWHs in children has also been suggested [32]. Strong linearity was observed for tinzaparin and enoxaparin calibration curves, with a slight bias towards lower anti-FXa activity with tinzaparin. However, the difference was not significant. With the present fluorogenic anti-FXa assay, the reaction rates for enoxaparin were marginally higher than for tinzaparin, but the differences were not statistically significant as previously reported in the literature [32].

Danaparoid has the greatest ratio of anti-FXa:anti-FIIa ($\geq 22:1$) amongst the anticoagulants evaluated and inhibits FXa to a much greater extent than FIIa [4]. The anti-FXa assay is considered to be the only adequate assay for monitoring danaparoid. In the present study, it was established that the fluorogenic anti-FXa assay was capable of detection and discrimination of danaparoid up to 1 U/ml where it demonstrated good reproducibility with CVs of less than 7%, in addition to good linearity between 0.2 and 1 U/ml. Danaparoid cannot be measured using PT, APTT or thrombin clotting time. However, there is a requirement to monitor it in patients with elevated levels of creatinine in serum [4]. A study compared the APTT, ACT and anti-FXa assays for monitoring danaparoid during cardiovascular operations [33]. In spiked samples, APTT and ACT were prolonged in the presence of increasing concentrations of danaparoid. However, in patient samples, both assays were insensitive to significant changes in danaparoid levels. The present assay needs to undergo evaluation in patient samples before its usefulness can be fully determined.

In conclusion, a novel fluorogenic anti-FXa assay has been developed which is capable of measuring the impact on the anti-FXa activity of therapeutic concentrations of UFH, tinzaparin, enoxaparin and danaparoid. The assay used 4 nM exogenous FXa and

0.9 μ M Peflafluor FXa substrate and was performed in commercial human pooled plasmas. This single assay configuration resulted in detection ranges of 0–1 U/ml for UFH, 0–0.6 U/ml tinzaparin, 0–0.8 U/ml enoxaparin, and 0–1 U/ml for danaparoid with discrimination between doses of 0.2 U/ml in nearly all cases and typical CVs below 7%.

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