

2012

Effects Of Four Commercially Available FXa Proteins On The Fluorogenic Anti-FXa Assay When Monitoring Unfractionated Heparin

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Recommended Citation

Harris, I. et al (2012) Effects of four commercially available FXa proteins on the fluorogenic anti-FXa assay when monitoring unfractionated heparin. *Blood Coagulation & Fibrinolysis*, 2012 Jan;23(1):98-103. doi:10.1097/MBC.0b013e32834ddf4d

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Funder: Enterprise Ireland

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Editorial Manager(tm) for Blood Coagulation & Fibrinolysis
Manuscript Draft

Manuscript Number: BCF-051306

Title: Effects of four commercially available FXa proteins on the fluorogenic anti-FXa assay when monitoring unfractionated heparin

Article Type: Short Communication

Keywords: FXa; bovine; human; fluorogenic anti-FXa assay; UFH; anticoagulant monitoring

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Manuscript Region of Origin: IRELAND

Abstract: Four commercially available Factor Xa (FXa) reagents were evaluated in a fluorogenic anti-FXa assay. The four reagents - of which three were of human origin and the fourth was bovine - were compared in terms of the resulting assay dynamic ranges, lag times, CV and R² values, sum of squares as well as their sensitivity to unfractionated heparin (UFH) within the therapeutic range of 0-1.2 U mL⁻¹. Based on a balance of performance characteristics, an optimum reagent was selected which had the best combination of all parameters. The best performing serine endopeptidase in the fluorogenic anti-FXa assay was found to be bovine in nature and in liquid form. The resulting assay was very sensitive within the dynamic range of 0-1.2 U mL⁻¹ showing a linear semi-logarithmic dose-response calibration curve with an R² of 0.99 and CVs <7 %. Of the three human FXa evaluated, the lyophilised human FXa in the absence of stabilizers was demonstrated to be the best performing human FXa reagent. It showed similar behaviour to the bovine reagent in terms of lag time values and dynamic range but with reduced sensitivity. CVs and R² values were ≤ 2 % and 0.95, respectively, when considering all points in the linear regression fit.

Submission to:

Journal name: BLOOD COAGULATION AND FIBRINOLYSIS

Title of paper: EFFECTS OF FOUR COMMERCIALY AVAILABLE FXa PROTEINS ON THE

Date submitted: 18/03/11 FLUOROGENIC ANTI-FXa ASSAY WHEN MONITORING

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Effects of four commercially available FXa proteins on the fluorogenic anti-FXa assay when monitoring unfractionated heparin

“Running head”: FXa and monitoring of unfractionated heparin

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Sources of support: This work was supported by Enterprise Ireland under Grant No. TD/2009/0124.

Abstract

Four commercially available Factor Xa (FXa) reagents were evaluated in a fluorogenic anti-FXa assay. The four reagents - of which three were of human origin and the fourth was bovine - were compared in terms of the resulting assay dynamic ranges, lag times, CV and R^2 values, sum of squares as well as their sensitivity to unfractionated heparin (UFH) within the therapeutic range of 0-1.2 U mL⁻¹. Based on a balance of performance characteristics, an optimum reagent was selected which had the best combination of all parameters. The best performing serine endopeptidase in the fluorogenic anti-FXa assay was found to be bovine in nature and in liquid form. The resulting assay was very sensitive within the dynamic range of 0-1.2 U mL⁻¹ showing a linear semi-logarithmic dose-response calibration curve with an R^2 of 0.99 and CVs <7 %. Of the three human FXa evaluated, the lyophilised human FXa in the absence of stabilizers was demonstrated to be the best performing human FXa reagent. It showed similar behaviour to the bovine reagent in terms of lag time values and dynamic range but with reduced sensitivity. CVs and R^2 values were ≤ 2 % and 0.95, respectively, when considering all points in the linear regression fit.

Keywords: FXa; bovine; human; fluorogenic anti-FXa assay; UFH, anticoagulant monitoring

Introduction

Anticoagulant monitoring is necessary as overmedication using drugs such as warfarin or heparins may lead to excessive uncontrolled bleeding, while under dosage of these drugs can hamper their function in the prevention of thrombus formation [1]. Unfractionated heparin (UFH) is a widely utilized parenteral anticoagulant drug which functions to bind to the naturally occurring anticoagulant protein antithrombin (AT) to catalyse AT inhibition of thrombin and Factor Xa (FXa) in addition to other serine endopeptidases [2].

Current methods of anticoagulant monitoring focus on clot-based assays. These assays, including the activated partial thromboplastin time (APTT) and activated clotting time (ACT), have several disadvantages [3,4] but continue to be used despite the fact that they lack standardization due to different reagents and instrumentation available for performing the wide range of commercially available tests [5,6]. In addition, the APTT and ACT can be affected by a number of coagulopathies (i.e. deficiencies of certain coagulation factors) [7].

Alternative anticoagulant monitoring tests include anti-FXa assays which are sensitive to UFH. The most commonly used are chromogenic anti-FXa assays which are based on a colorimetric end-point measurement. Unfortunately, there are also some drawbacks to chromogenic anti-FXa assays such as the cost, lack of assay uniformity and limited published information on their use [8-10]. Moreover, these assays measure the change in absorbance of the sample, which complicates measurements in whole blood or platelet-rich-plasma (PRP) samples [11]. Therefore, consideration has been given to the development of fluorogenic anti-FXa assays, as these could allow for monitoring all forms of heparins in a range of sample types [3]. The principle behind the fluorogenic anti-FXa assay previously developed is that UFH, endogenous AT and FXa (added in excess) form a complex. The remaining FXa is free to cleave the fluorogenic substrate at a specific site, thus releasing the corresponding fluorophore which is inversely proportional to the concentration of UFH present in the sample.

The purpose of this study was to compare the performance of four different commercially available FXa preparations in a previously-developed fluorogenic anti-FXa assay, and assess them in terms of resulting assay dynamic ranges, lag times, CV and R^2 values, sum of squares as well as their sensitivity to UFH within the therapeutic range of $0-1.2 \text{ U mL}^{-1}$.

Materials and methods

Reagents

Water (molecular biology reagent) and HEPES (minimum 99.5% titration) were purchased from Sigma-Aldrich (Dublin, Ireland). Filtered HEPES was prepared at a concentration of 10 mM (pH 7.4). A 100 mM filtered stock solution of CaCl₂ from Fluka BioChemika (Buchs, Switzerland) was prepared from a 1 M CaCl₂ solution.

Purified human/bovine FXa serine endopeptidase (code number: EC 3.4.21.6) was purchased from four different companies in lyophilized or liquid form, aliquoted into 10 µL and stored at -20 °C until further use. The four FXa reagents used were as follows: Human FXa from Hyphen Biomed (Neuville-Sur-Oise, France) in lyophilized form was reconstituted in 500 µL water to give a final concentration of 4400 nM; Human FXa from Enzyme Research Laboratories Ltd. (Indiana, USA) in lyophilized form was reconstituted in 584 µL water to give a final concentration of 51 µM; Bovine FXa from Sigma-Aldrich (Steinheim, Germany) was received in liquid form at a concentration of 117.4 µM; Human FXa from Haematologic Technologies Inc. (Vermont, USA) was received in liquid form at a concentration of 206.5 µM. Table 1 summarises the characteristics of the four FXa preparations studied. Stocks were subsequently used to prepare a working concentration of 12 nM FXa in 10 mM HEPES buffer for use in the fluorogenic anti-FXa assay.

The fluorogenic substrate methylsulfonyl-D-cyclohexylalanyl-glycyl-arginine-7-amino-4-methylcoumarin acetate (Peflafluor 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. Dilutions from 10 mM stock solutions down to 10 µM were freshly prepared with water when needed. Subsequent dilutions to 2.7 µM were prepared in 10 mM HEPES. Tubes were covered with aluminium foil to protect from exposure to light.

Human pooled plasma was purchased from Helena Biosciences Europe (Tyne and Wear, UK). Plasma was received in lyophilized form. It was reconstituted in 1 mL water and left to stabilize for a minimum of 20 min at room temperature before use. UFH obtained from bovine lung tissue was acquired from Sigma-Aldrich (St Louis, MO). A stock solution of 100 U mL⁻¹ was used to prepare all subsequent dilutions within the therapeutic range of 0–1.2 U mL⁻¹.

(Table 1)

Apparatus

Fluorescence intensities were measured on an Infinite M200 microplate reader from Tecan Group Ltd. (Männedorf, Switzerland) equipped with a UV Xenon flashlamp. Assays were performed in flat, black-bottom 96-well polystyrol FluorNunc™ microplates from Thermo Fisher Scientific (Roskilde, Denmark).

Fluorogenic assay procedure

The experimental protocol previously developed by Harris et al. [3] was used. Briefly, to establish optimal assay concentrations, Peflafluor FXa fluorogenic substrate and FXa from Hyphen Biomed were titrated over a range of concentrations. The optimized fluorogenic anti-FXa assay consisted of 4 nM FXa. A stock solution of 12 nM FXa in 10 mM HEPES was prepared in each case. Stocks were then incorporated into the fluorogenic assay with freshly prepared fluorogenic substrate as indicated above. The reactions were carried out using UFH concentrations from 0 to 1.2 U mL⁻¹ every 0.2 U mL⁻¹ in human pooled plasma. The excitation/emission pair of 342/440 nm was used to monitor the formation of the fluorophore 7-amino-4-methylcoumarin (AMC). The reaction rate (slope), which was 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 anticoagulant concentration. Lag times were calculated by extrapolation of the linear portion of the progression curve to its intersection with a parallel line to the x-axis that goes through all points between the beginning of the experiment and the first recorded measurement.

Statistical analysis

All graphs were plotted using SigmaPlot 8.0. Statistical analysis was carried out using SPSS 17.0 software. Log transformations were applied to all reaction rates for data normalization. Intra-assay variability was determined using one-way analysis of variance (ANOVA) and a result of p<0.05 was considered statistically significant. If significance was observed, Tukey's post-hoc test was performed. Homogeneity of variance was a requirement of statistical analysis and was assessed by the Levene's test (p<0.05).

Results and discussion

The optimized fluorogenic anti-FXa assay was found to be 4 nM FXa and 0.9 μ M Pefafleur FXa, which was capable of statistically differentiating the log of the slope values (i.e. $\log(dF/dt)$) in commercial human pooled plasma from 0 to 1 U mL⁻¹ at intervals of 0.2 U mL⁻¹. Linear regression analysis within the statistically sensitive range was also calculated returning a calibration curve and correlation coefficient of $y = -0.51x + 2.17$ and $R^2 > 0.98$, respectively. Sum of squares resulted in a value of 0.05.

In the present study, a further three commercially available FXa serine endopeptidases were examined in the same fluorogenic anti-Xa assay and compared in terms of assay dynamic ranges, lag times, CV and R^2 values, sum of squares as well as their sensitivity to UFH within the therapeutic range of 0-1.2 U mL⁻¹. Three out of the four FXa proteins were of human origin while the remaining one was bovine.

As can be seen in all insets from Fig. 1 to Fig. 3, all reaction progress curves are similar to that described by Harris et al. [3] for the assay employing Hyphen Biomed FXa. As UFH concentration increased, lag times were extended and reaction rates were reduced. In the case of the FXa from Sigma-Aldrich (Fig. 1, inset), the reaction curves reached a plateau at approximately 25,000-28,000 arbitrary fluorescent units (F.U.) at all anticoagulant concentrations except for 1 and 1.2 U mL⁻¹ which did not level off. This increment in signal indicates an increase in fluorescence intensity of ca. 17,000 F.U. Lag time values increased with increasing UFH concentration from 70 s to 1790 s from 0.4 to 1.2 U mL⁻¹, respectively. A lag time was not observed when working at low UFH concentrations (i.e. 0-0.2 U mL⁻¹). Table 2 summarizes lag time values at all concentrations for all FXa reagents studied.

(Fig. 1) and (Table 2)

Intra-assay variability was calculated by means of statistical analysis of the log mean slope values obtained at all UFH concentrations. Statistical analysis of the data showed sensitivity of the assay up to 1.2 U mL⁻¹ UFH ($p < 0.05$). Standard deviations of triplicate measurements were within acceptable range as shown by CVs of no greater than 7 %, indicating good reproducibility. A linear regression was found between the $\log(dF/dt)$ and UFH concentration in the assay sensitive range 0-1.2 U mL⁻¹ (Fig. 1). The linear calibration curve equation was $y = -1.01x + 1.78$ with $R^2 = 0.99$. Sum of squares was found to be 0.99.

The reaction progress curve of the fluorogenic anti-FXa assay when using FXa from Enzyme Research Laboratories Ltd. (Fig. 2, inset) reached a plateau at approximately 27,000 F.U. independent of anticoagulant concentration, which indicates an increase in fluorescence intensity of ca. 16,000 F.U. The lag time values observed on this occasion varied from 430 s up to 1570 s when working at UFH concentrations of 0.4–1.2 U mL⁻¹ UFH (Table 2). No lag time was observed from 0–0.2 U mL⁻¹. These values indicate a slightly slower fluorogenic anti-FXa assay when compared with the assay in the presence of FXa from Sigma-Aldrich.

(Fig. 2)

The statistically sensitive range was established as 0–1.2 U mL⁻¹ UFH ($p < 0.05$), similar to the assay using FXa from Sigma-Aldrich. Reproducibility was found to be ≤ 2 %CV indicating good assay precision. The linear calibration curve equation taking into consideration all points in the statistically sensitive range of the log/lin plot (Fig. 2) was $y = -0.64x + 1.79$ and the R^2 value observed was 0.95. It should be noted that linearity in the early part of the semi-logarithmic dose-response curve (i.e. from 0–0.2 U mL⁻¹) is poor when compared with the rest of the concentration values as seen visually. In order to mathematically confirm this, sum of squares was performed in the presence and absence of the value at 0 U mL⁻¹. When considering the $\log(dF/dt)$ value at 0 U mL⁻¹, sum of squares resulted in a value of 0.20 while in its absence it decreased down to 0.11. These results indicated that the linear behaviour of the calibration curve could be largely improved when the $\log(dF/dt)$ value was not taken into consideration at 0 U mL⁻¹.

Fig. 3 (inset) shows the fluorescence response profile of the fluorogenic anti-FXa assay using FXa from Haematologic Technologies Inc. The fluorescence kinetic profile reached a plateau at approximately 27,000 F.U. at all UFH concentrations except at 1 and 1.2 U mL⁻¹; this corresponds to an increase in fluorescence intensity of ca. 11,500 F.U. The enzymatic reaction did not show any lag time between 0–0.2 U mL⁻¹ UFH. Nevertheless, the lag time values observed from 0.6–1.2 U mL⁻¹ UFH were the largest of the four FXa proteins varying from 860 s up to at least 3600 s. Taking into account Table 2, overall values indicate an increase in the following order: Hyphen Biomed < Sigma-Aldrich ~ Enzyme Research Laboratories Ltd. < Haematologic Technologies Inc.

(Fig. 3)

As can be seen in Fig. 3, the slope value at 1.2 U mL^{-1} could not be determined as the reaction did not take place during the time-frame of the experiment. Tukey's post-hoc test proved that the assay sensitivity ranged from $0-1 \text{ U mL}^{-1}$ UFH ($p < 0.05$) but no significant differences were observed between $0-0.2$ and $0.6-0.8 \text{ U mL}^{-1}$. CV values at all concentrations were $< 3.5\%$. The calibration curve of the assay sensitive range (Fig. 3) was fitted to the linear regression equation $y = -1.13x + 1.97$ with an R^2 of 0.96. Sum of squares resulted in a value of 0.47.

The four fluorogenic anti-FXa assays were compared considering different factors such as sensitivity to UFH, dynamic range, lag times, CV and R^2 values as well as the sum of squares. Table 3 summarises all parameters taken into account when analysing similarities and differences in the anti-FXa fluorogenic assay performance depending on the FXa employed.

(Table 3)

Sensitivity and dynamic ranges

Comparison of the four fluorogenic anti-FXa assays in terms of their intra-assay variability to UFH resulted in assays capable of measurement up to 1 U mL^{-1} ($p < 0.05$) when human FXa was sourced from Hyphen Biomed and Haematologic Technologies Inc. FXa from Hyphen Biomed is available in lyophilized state in the presence of a mixture of stabilizers, whereas FXa from Haematologic Technologies Inc. is in aqueous form using 50% (v/v) glycerol/water stabilizer solution. FXa obtained from Sigma-Aldrich or Enzyme Research Laboratories Ltd. increased the UFH sensitive range to 1.2 U mL^{-1} ($p < 0.05$). The FXa from Sigma-Aldrich is bovine in nature and comes in liquid state (50/30% glycerol/water) whereas the origin of the FXa from Enzyme Research Laboratories Ltd. is human and it exists as a lyophilized solid without stabilizers. It has been reported by Lai and Topp [12] that proteins in solid form are generally more stable than the respective aqueous formulations. On this occasion, the physical state of the formulation seems not to have a major effect on assay performance as assay dynamic ranges were independent of this property.

Another parameter to consider is protein source. All FXa proteins were human in origin except for the FXa obtained from Sigma-Aldrich which was bovine. As indicated by the manufacturers, all FXa reagents were derived from homogeneous/highly purified FX activated by Russells' Viper Venom (RVV). Thus, it could be speculated that significant differences can be found in the peptide sequences of the proteins depending on the source, which may account for differing performances. For instance, Edwards et al. [13] compared human and rabbit coagulation factor X in terms of different parameters, and concluded that significant structural and physiological differences exist between both FXs. The activation of FX to FXa by RVV was found to be 30% slower for the rabbit protein than for the human FX. Moreover, lower chromogenic substrate cleavage rates were observed for the rabbit FX than those by human protein. However, Chen et al [14] characterised and compared both porcine and human FVII and FX finding similarities in nucleotide, amino acid sequences and three-dimensional structure. Conversely, the activity of porcine FX was 1.49-fold of average human FX activity. Similar to this were other findings in the literature, which indicated that the K_m value for RVV activation of human FX was 25-fold lower than that for bovine FX [15, 16].

Based on these previous observations, it can be suggested that depending on the FXa's source different FXa's activities and affinities for other compounds may indicate possible variations at FXa's active and binding sites. For example, the divalent metal ion relationship with human or bovine FX was studied and results showed that Mn^{2+} could be substituted for Ca^{2+} when bovine FXa was employed. Contrary to this, human FXa protein could not be replaced with Mg^{2+} or Mn^{2+} [16, 17].

Assay sensitivity is given by the slope values of the linear semi-logarithmic dose-response calibration curves. The larger its negative value the more sensitive the assay results. As seen in Table 3, FXa from both bovine Sigma-Aldrich and human Haematologic Technologies Inc. showed approximately two-fold higher sensitivity to UFH than the other two human lyophilised FXa proteins.

Sum of squares, R^2 and CV values

It should be noted that all FXa reagents investigated exhibited linear calibration curves with R^2 values of 0.95, 0.95, 0.96 and 0.99, the last two values correspond to FXa from Haematologic Technologies Inc. and Sigma-Aldrich, respectively. Another criterion for whether a fit is reasonable it is the sum of squares. Values were calculated for all reagents increasing in the following order: Hyphen Biomed (0.05) <

Enzyme Research Laboratories Ltd. (0.20) < Haematologic Technologies Inc. (0.47) < Sigma-Aldrich (0.99). The smaller the sum of squares the better the fit but also, something to consider is that the sum of squares will grow with the size of the data collection. Therefore, considering our results and data set, FXa from Hyphen Biomed seems to fit better the data to a linear calibration curve than FXa from Haematologic Technologies Inc. In the case of FXa from Enzyme Research Laboratories Ltd. and Sigma-Aldrich, the former showed lower sum of squares than the latter although linearity in the early part of the semi-logarithmic dose-response curve was poor as previously indicated. Hence, FXa bovine in origin seems to offer more assay precision.

Moreover, CVs were <5 % for FXa from Enzyme Research Laboratories Ltd. and Haematologic Technologies Inc. while <7 % for the other two human proteins. Another parameter to take into account for the different behaviour of the four FXa reagents is purity. Haematologic Technologies Inc. as well as Enzyme Research Laboratories Ltd. reported a purity value > 95% tested by SDS-PAGE analysis, but accurate information on the other commercial FXa proteins was unavailable. It is well known that protein purity influences protein activity [18,19]. Therefore, reproducibility and the affinity properties of the different commercial FXa reagents towards the fluorogenic substrate can also be related to their purity values.

To summarise, lyophilised human FXa from Hyphen Biomed returned the shortest lag time values with a statistically sensitive UFH range up to 1 U mL^{-1} , CV and R^2 values of <7 % and 0.95, respectively. On the other hand, lyophilised human FXa from Haematologic Technologies Inc. showed the largest lag time values and was one of the most sensitive assays up to 1 U mL^{-1} . CV and R^2 values were <3.5 % and 0.96, respectively. Both bovine and human FXa proteins from Sigma-Aldrich and Enzyme Research Laboratories Ltd. demonstrated similar behaviour in terms of lag time values and a more extended UFH sensitive range from $0\text{-}1.2 \text{ U mL}^{-1}$. Nevertheless, liquid bovine FXa from Sigma-Aldrich resulted in the most sensitive fluorogenic anti-FXa assay, and a more precise linear calibration curve with an R^2 value of 0.99 considering all data points.

From these results, it can be concluded that after taking into consideration all different factors such as assay sensitivity to UFH, dynamic range, lag times, sum of squares as well as CV and R^2 values, the best performing FXa reagent in the fluorogenic anti-FXa assay was bovine in nature and in liquid form

(Sigma-Aldrich), followed by lyophilised human FXa in the absence of stabilisers (Enzyme Research Laboratories Ltd).

Acknowledgements

We would like to extend our thanks to Dr Michael Parkinson at Dublin City University (DCU) for his very helpful advice in the statistical analysis.

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Legends for illustrations

Fig. 1 Semi-logarithmic dose-response calibration curve of UFH in pooled plasma in the fluorogenic anti-FXa assay with FXa sourced from Sigma-Aldrich. The linear calibration curve obtained was $y = -1.01x + 1.78$, $R^2 = 0.99$. Inset: Fluorescence intensity vs. time for the fluorogenic assay run within the range 0–1.2 U mL⁻¹ UFH in human pooled commercial plasma. UFH concentration increases from left to right (n=3)

Fig. 2 Semi-logarithmic dose-response calibration curve of UFH in pooled plasma in the fluorogenic anti-FXa assay with FXa sourced from Enzyme Research Laboratories Ltd. The linear calibration curve obtained, when considering all points, was $y = -0.64x + 1.79$, $R^2 = 0.95$. Inset: Fluorescence intensity vs. time for the fluorogenic assay run within the range 0–1.2 U mL⁻¹ UFH in human pooled commercial plasma. UFH concentration increases from left to right (n=3)

Fig. 3 Semi-logarithmic dose-response calibration curve of UFH in pooled plasma in the fluorogenic anti-FXa assay with FXa sourced from Haematologic Technologies Inc. The linear calibration curve obtained was $y = -1.13x + 1.97$, $R^2 = 0.96$. Inset: Fluorescence intensity vs. time for the fluorogenic assay run within the range 0–1.2 U mL⁻¹ UFH in human pooled commercial plasma. UFH concentration increases from left to right (n=3)

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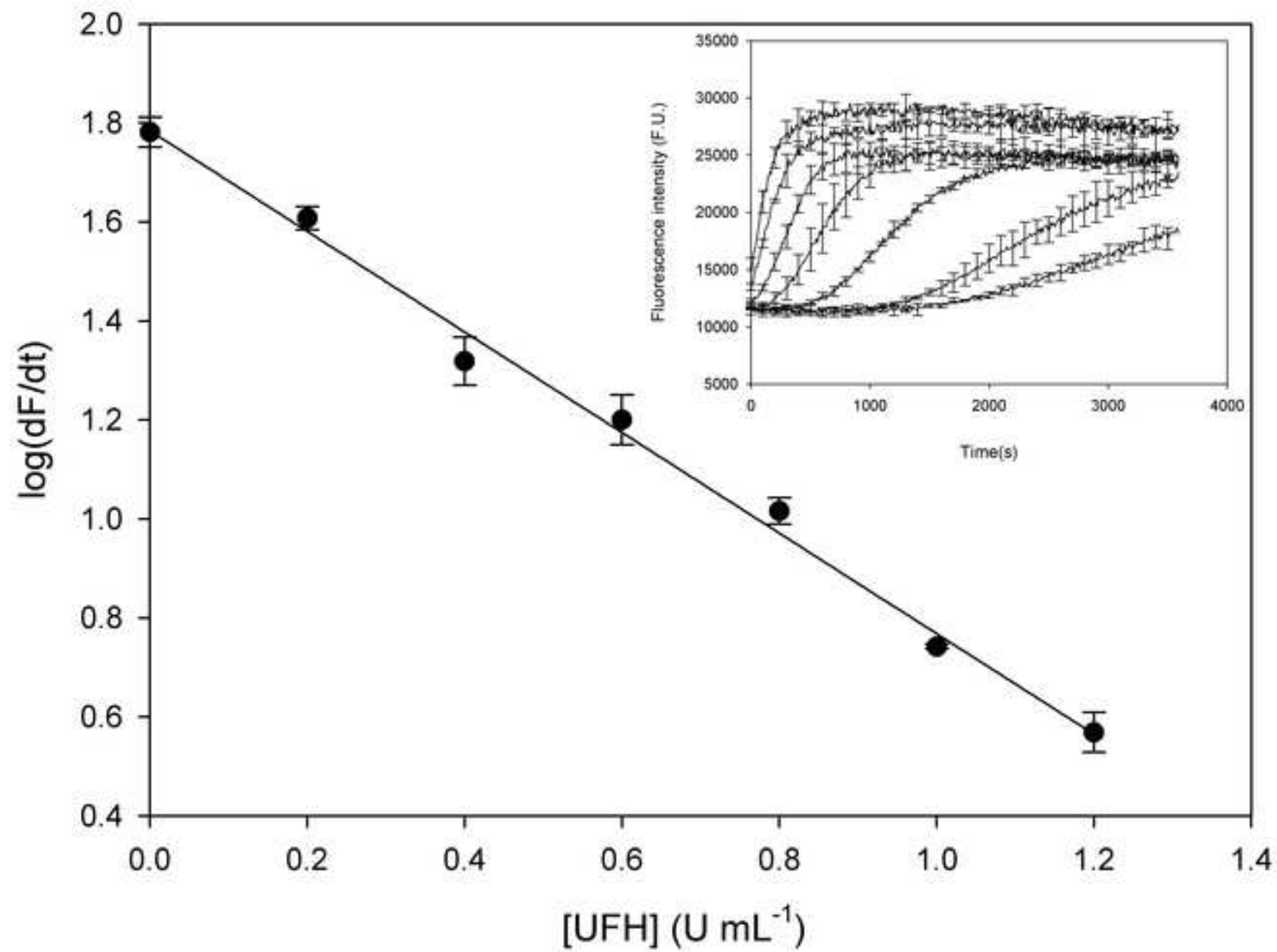


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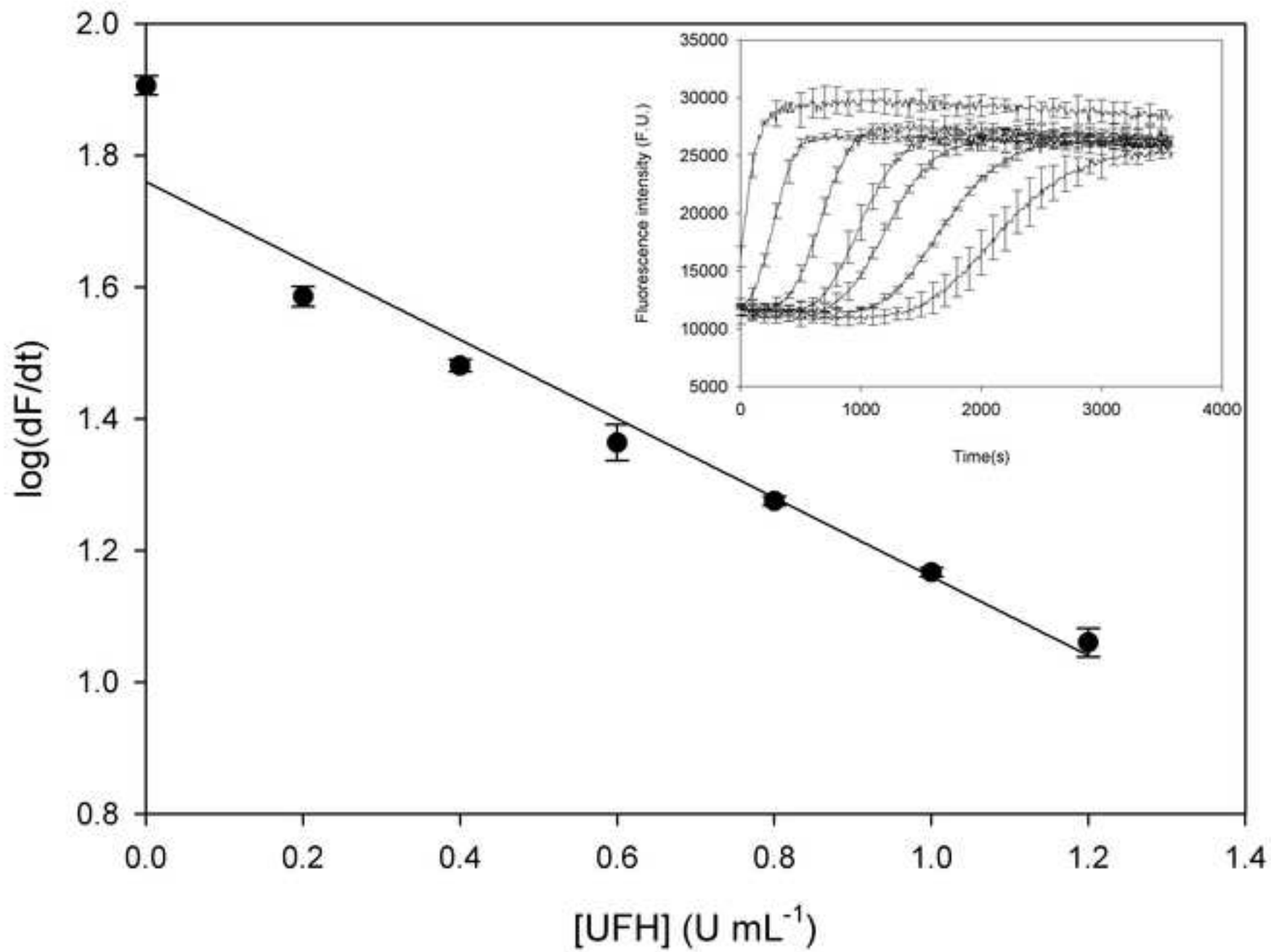


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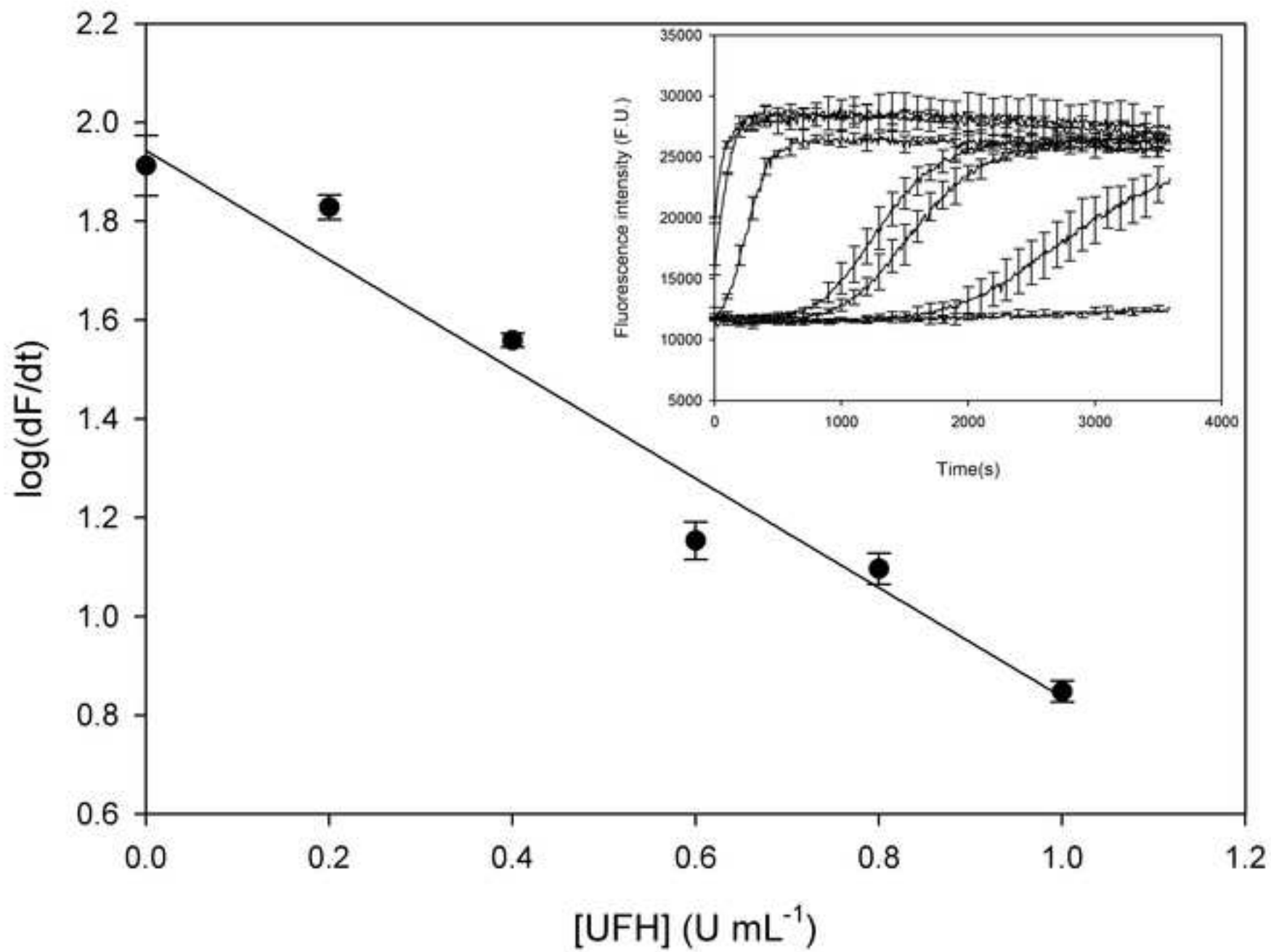


Table 1 Properties of four commercial FXa reagents

Manufacturer^a	Source	Physical state	Stabilizers	Purity
Hyphen Biomed	Human	Lyophilized	Glycine, Prionex, PEG 6000, sodium chloride	-
Enzyme Research Laboratories Ltd.	Human	Lyophilized	none	> 95%
Haematologic Technologies Inc.	Human	Liquid	50% (v/v) glycerol/H ₂ O	> 95%
Sigma-Aldrich	Bovine	Liquid	50/30% (v/v) glycerol/H ₂ O	-

^a All FXa reagents were reported to have Mw of ~ 46 KDa

Table 2 Lag time values and standard deviation of the fluorogenic anti-FXa assay in the presence of four different commercial FXa proteins within the range of 0–1.2 U mL⁻¹ UFH

[UFH] (U mL ⁻¹)	Lag time values ^b (s)			
	Hyphen Biomed	Sigma-Aldrich	Enzyme Research Laboratories Ltd.	Haematologic Technologies Inc.
0	0	0	0	0
0.2	160 ± 10	0	0	0
0.4	225 ± 10	70 ± 25	430 ± 25	70 ± 10
0.6	390 ± 20	200 ± 60	700 ± 60	860 ± 100
0.8	430 ± 20	580 ± 0	860 ± 20	1140 ± 105
1	660 ± 30	1320 ± 110	1230 ± 0	2050 ± 195
1.2	850 ± 10	1790 ± 30	1570 ± 100	3600 ± 0

^b Average of triplicate measurements (n=3)

Table 3 Summary table of the fluorogenic anti-FXa assay in the presence of four different commercial FXa proteins

Parameters	Hyphen Biomed	Sigma-Aldrich	Enzyme Research Laboratories Ltd.	Haematologic Technologies Inc.
Sensitivity (log(dF/dt) mL U ⁻¹)	-0.5	-1	-0.6	-1.1
Dynamic range (U mL ⁻¹)	0-1	0–1.2	0–1.2	0-1
CV (%)	<7	<7	≤2	<3.5
R²	0.95	0.99	0.95	0.96
Sum of squares	0.05	0.99	0.20	0.47