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Quantification of unfractionated heparin in human plasma and whole blood by means of novel fluorogenic anti-FXa assays

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Abstract

Novel and sensitive plate-based fluorogenic anti-factor Xa (FXa) assays were investigated to quantify unfractionated heparin (UFH) in human plasma and whole blood within the therapeutic ranges of 0-1.6 U/mL and 0–0.8 U/mL, respectively. Two fluorogenic anti-FXa assay methods were defined for low (0-0.6 U/mL) and high (0.6-1.2 U/mL) pharmacologically relevant UFH concentration ranges in pooled human plasma. In both cases significant differences were observed at intervals of 0.2 U/mL ($P<0.05$). The semi-logarithmic plots of the calibration curves in the low and high UFH range were both fitted to linear regressions with correlation coefficients of 0.96 and >0.99, respectively. The assay was also optimized for whole blood which was capable of differentiating UFH concentrations at intervals of 0.2 U/mL ($P<0.05$) in the range of 0-0.4 U/mL. The statistically different results were fitted to a linear regression with a correlation coefficient of >0.99. The results obtained in this study could assist diagnostic laboratories towards improved monitoring of UFH therapy.

Keywords: fluorogenic anti-FXa assay; whole blood; plasma; UFH; monitoring.

1. Introduction

The anticoagulant drugs market is believed to increase to over €9 billion in 2014 from €6 billion in 2008 [1]. Unfractionated heparin (UFH)[1] has been the parenteral anticoagulant of choice for more than 50 years [2-4]. Even though new anticoagulant drugs inhibiting thrombin (30%) and factor Xa (FXa) (70%) are under clinical development [5-7], UFH continues to be administered for short-term prophylaxis because it is effective, inexpensive, and a protamine sulfate antidote exists to rapidly reverse bleeding [8].

Common laboratory monitoring of UFH is carried out by traditional coagulation tests, such as the clot-based activated partial thromboplastin time (APTT) [9], chromogenic anti-FXa assays [10], and the activated clotting time (ACT). Due to some limitations and drawbacks associated with APTT and ACT, it has been recommended to calibrate the therapeutic APTT range in seconds to the reference anti-FXa range of 0.3-0.7 anti-FXa U/mL [11].

Fluorescent detection has been investigated over the last few years as an alternative technique to clotting and chromogenic assays. The high sensitivity that this optical measurement can offer [12] along with its ability to be adapted to a broad range of sample matrices, makes it an interesting technique to investigate. Many fluorophores and labelling chemistries are

[1] **Abbreviations:** UFH, unfractionated heparin; FXa, factor Xa; APTT, activated partial thromboplastin time; ACT, activated clotting time; AMC, 7-amino-4-methylcoumarin; SN-7, Mes-D-LGR-ANSN $(C_2H_5)_2$ fluorogenic substrate; ANSN, 6-amino-1-naphthalene-sulfonamide; DCU, Dublin City University; ANOVA, analysis of variance; afu, arbitrary fluorescence units; TEG, thromboelastography; TAS HMT, thrombolytic assessment system heparin management test; TG, thrombin generation.

available for different coagulation proteases such as factor VIIa [13] and factor VIIa-tissue factor complex [14], thrombin [15], factor VIII [16], factor IX aβ [13, 17], factor Xa [13, 15], factor XIa [18], factor XIIa [19] and factor XIII [20]. In particular, fluorogenic substrates have been used to measure thrombin generation over the last ten years to investigate the effects of UFH in platelet-poor-plasma (PPP) [21] and platelet-rich-plasma (PRP) [22].

To the best of our knowledge, few publications to date have evaluated the use of fluorogenic substrates in the development of anti-FXa assays for monitoring anticoagulant therapy in human plasma [23, 24] with none reported for use in whole blood. A novel and sensitive plate-based fluorogenic anti-FXa assay in human plasma was recently investigated to monitor UFH therapy using one of two commercially available peptide substrates for FXa based on a 7-amino-4-methylcoumarin (AMC) fluorescent reporter group [23]. In this study, it was sought to develop novel fluorogenic anti-FXa assays to monitor therapeutic UFH concentrations in both human pooled plasma and whole blood using the only other commercially available FXa fluorogenic substrate.

2. Materials and methods

2.1. Reagents

Water (molecular biology reagent), HEPES (minimum 99.5% titration), sodium citrate tribasic dihydrate (ACS reagent, ≥ 99.0%) and citric acid monohydrate (ACS reagent, 99.0-102.0%) were purchased from Sigma-Aldrich (Dublin, Ireland). Filtered HEPES (pH 7.4; 10 mM) was prepared and both sodium citrate and citric acid were made up to 0.1 M. Citrate-citric acid buffer solution was prepared at 3.8% sodium citrate and adjusted to ca. pH 5.5 with 0.1 M citric acid. 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

Mes-D-LGR-ANSN $(C_2H_5)_2$ (SN-7), containing the fluorescent reporter group 6-amino-1-naphthalene-sulfonamide (ANSN), was acquired from Haematologic Technologies Inc. (Vermont, USA). Stock solutions of 10 mM in DMSO were stored at -20 °C and also protected from light with aluminum foil. Dilutions of stock solutions were performed with 10 mM HEPES. The kinetic constants of SN-7 fluorogenic substrate, which refer to the cleavage by the endopeptidase FXa, were provided by the supplier as follows: K_m = 125 µM; k_{cat} = 36 s^{-1}; k_{cat}/K_m = 290,000 M^{-1} s^{-1}. Purified human FXa (serine endopeptidase; code number: EC 3.4.21.6) was obtained from Hyphen BioMed (Neuville-Sur-Oise, France). UFH obtained from bovine lung tissue was acquired from Sigma-Aldrich (St Louis, MO). Human pooled plasma was purchased from Helena Biosciences Europe (Tyne and Wear, UK). Lyophilized 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. Blood sampling

Human blood was locally obtained from one smoking and five non-smoking healthy volunteers (30 – 40 years of age; 3 males and 3 females), who had not ingested any pharmacologically active substances prior to the experiment. Informed consent was granted by all volunteers and the study was approved by the Dublin City University (DCU) ethics committee. Samples were drawn through antecubital venipuncture at the School of Health and Human Performance in DCU. 10 mL of venous blood was collected into plastic 20 mL sterile BD Luer-Lok™ syringes from Becton, Dickinson and Company (Drogheda, Ireland), containing 3.8% sodium citrate at a ratio of 1:10 in blood using sterile Venisystems™ Butterfly®-19 needles from Abbott (Sligo, Ireland).

2.3. Apparatus

Absorbance and fluorescence intensities were measured on an Infinite M200 microplate reader from Tecan Group Ltd. (Männedorf, Switzerland) equipped with a UV Xenon flashlamp. Flat, black-bottom 96-well polystyrol FluorNunc™ microplates fromThermo Fisher Scientific (Roskilde, Denmark) were used for fluorescence measurements, and flat, transparent 96-well Greiner® microplates from Greiner Bio-One (Gloucestershire, United Kingdom) for absorbance readings.

2.4. Absorbance and emission spectra of whole blood samples

The total volume of samples in the absorbance and emission fluorescence experiments was 150 µL, which corresponds to the same final volume as used in the fluorogenic anti-FXa assay. Human blood samples were diluted 1:150 in water and their absorption spectra were measured at 2 nm intervals with the Infinite M200 microplate reader from 280 to 1000 nm. Background fluorescence emission spectra were evaluated at the excitation wavelength of 352 nm, which corresponds to that of the ANSN-based fluorogenic substrate, starting at 400 nm up to 850 nm at 2 nm reading intervals. Whole blood was diluted 1:3 in water. All samples were scanned at 37 ºC and after the assay was run.

2.5. Optimization of fluorogenic anti-FXa assays

Measurements were carried out in reconstituted citrated human pooled plasma and citrated human whole blood. All assays using human whole blood were initiated within 15 min of collecting the blood samples. The same experimental protocol was used for both matrices, the only difference being the titration range of both FXa and SN-7 fluorogenic substrate. In the presence of citrated human pooled plasma, FXa and the ANSN-based fluorogenic substrate were titrated within the range of 0.1-200 nM and 8.3-75 µM (K_m = 125 µM), respectively. In

the case of citrated human whole blood, FXa and the fluorogenic substrate were titrated from 300 to 500 nM and from 75 to 125 µM, respectively. The experimental protocol was as follows: samples consisting of 6.25 µL of 100 mM $CaCl_2$, 43.75 µL of pooled plasma or citrated whole blood, and 50 µL of FXa (within the titration ranges) were incubated at 37 °C for 3 min and shaken for the first 150 s. The reaction was started by adding 50 µL of ANSN-based fluorogenic substrate within the titration ranges previously described. Samples within wells were mixed with the aid of orbital shaking at 37 °C for 30 s. Finally, immediately after shaking, fluorescence measurements were recorded at 37 °C for 60 min with 20 µs integration time. Fluorescence excitation was at 352 nm and emission was monitored at 470 nm, corresponding to the excitation/emission wavelengths of the ANSN fluorophore. All measurements were carried out in triplicate. Following optimization of assay conditions, pooled commercial plasma and whole blood samples were spiked with therapeutically relevant concentrations of UFH from 0–1.6 U/mL and from 0-0.8 U/mL, respectively. 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 anticoagulant concentration.

2.6. Software and statistical analysis

All graphs were plotted using SigmaPlot 8.0. Statistical analysis was carried out using SPSS 17.0 software. Logarithmic transformation was applied to all reaction rates for data normalization**.** Intra-assay differences within the anticoagulant concentration range were compared using one-way analysis of variance (ANOVA), with subsequent post-hoc analysis performed (Tukey's test) if significance was observed. A result of $p<0.05$ was considered statistically significant.

3. Results

3.1. Human plasma

In the first part of the study in plasma, optimization of the fluorogenic anti-FXa assay was undertaken by titrating FXa and the SN-7 fluorogenic substrate within the range of 0.1-100 nM and 8.3-33.3 μM (K_m = 125 μM), respectively. The best performing assay was selected in terms of lag time and reaction rate values, as well as an optimal increase in fluorescence response. It was found that 100 nM FXa and 25 μM fluorogenic substrate fulfilled all these performance requirements. Subsequently, the fluorogenic anti-FXa assay developed was analysed in human pooled plasma spiked with UFH from 0 to 1.6 U/mL UFH every 0.2 U/mL. The reaction progress curves shown in the inset of Fig. 1 showed decreasing reaction rates and increasing lag times with increasing UFH concentration. This indicates that the amount of FXa left over after the inhibition of FXa by the complex formation of antithrombin-UFH, hence the fluorphore release, is inversely proportional to the concentration of UFH present in the reaction mixture.

Reaction rates were calculated and data normalization was performed by applying the $\log_{10}$-transformation to slope values (Fig. 1 and Table 1). Intra-assay variability was determined 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 1.6 U/mL UFH ($p<0.05$) except that 0.6 and 1 U/mL, and 1.2 and 1.4 U/mL, there were not significantly different from one another. Table 1 outlines the mean of the log of the slopes, their corresponding standard deviation (SD) and %CV values. As can be seen, all CVs <2.5% indicating good reproducibility of results.

Linear regression analysis was used to assess the behaviour of the log of the slopes with increasing UFH concentration. Even though the dynamic assay range was previously indicated as 0-1.6 U/mL UFH, the entire range was not considered for the linear calibration

curve as the linear fit returned a poor regression coefficient. From the data, a linear regression was found within the assay sensitive range 0-0.6 U/mL ($y = -1.37x + 1.80$; R^2 of 0.96).

In light of these results, it was considered appropriate to re-optimize the fluorogenic anti-FXa assay to investigate if a high range of UFH concentration could be monitored. Therefore, in the second part of the study in plasma, 200 nM FXa was titrated with the SN-7 fluorogenic substrate over the range of 25 to 75 μM. The requirements for the best performing assay were the same as previously indicated. Thus, 200 nM FXa and 75 μM SN-7 fluorogenic substrate were found to be the optimal assay concentrations. The assay was tested within the UFH range of 0.6-1.6 U/mL. As can be seen in the inset of Fig. 2, the reaction progress curve showed negligible lag time values, fluorescence intensity reached the upper limit of the instrument and differences in slope values are shown at different UFH concentrations. Fig. 2 illustrates the log of the slopes at all UFH concentrations studied. Statistical analysis demonstrated that UFH concentrations at 0.2 U/mL intervals were statistically different from one another ($p<0.001$) from 0.6 up to 1.2 U/mL. Table 1 summarises the log (dF/dt), SD and %CV values. As can be seen, reproducibility was consistent throughout the experiment with CVs <2%. Linear regression was carried out within the statistically sensitive range and the linear calibration curve equation was $y = -0.43x + 1.90$ with a correlation coefficient of >0.99.

3.2. Whole blood

3.2.1. Absorption and emission spectra

The absorption and emission fluorescence spectra of blood samples from six donors were measured in this study to investigate the effect of whole blood background fluorescence in the fluorogenic anti-FXa assay. Fig. 3 shows the absorption spectra of whole blood from the

six volunteers. As can be seen, the main absorption peaks appear at ~ 280, 345, 415, 541, and 576 nm, with the maximal absorption band at 415 nm. Differences in absorbance intensities can be observed between individuals, which can be attributed to intrinsic donor characteristics.

In order to investigate the whole blood autofluorescence, the emission spectra were evaluated at the excitation wavelength of 352 nm, which corresponds to that of the ANSN fluorophore (Fig. 4). A unique, but broad peak was observed at ca. 470 nm with a fluorescence intensity value of 500-600 arbitrary fluorescence units (afu), which represents only 1% of the total fluorescence range of the instrument (i.e. 0-60,000 afu). Thus the ANSN-based fluorogenic substrate proves to be a suitable label due to the almost negligible background emission fluorescence of whole blood at the wavelength excitation/emission pair of 352/470 nm.

3.2.2. Fluorogenic anti-FXa assay

Despite the very low emission fluorescence background shown by whole blood when exciting at 352 nm, the ANSN-based fluorogenic anti-FXa assay had to be re-optimized due to the very low increment in fluorescence signal. FXa and the SN-7 fluorogenic substrate were titrated within the range of 300-500 nM and from 75 to 125 μM, respectively. Whole blood samples from 6 donors were spiked with UFH concentrations within the range of 0-0.8 U/mL and optimization was assessed as previously described for the plasma assay. The best performing assay was found to be 350 nM FXa and 87.5 μM SN-7 fluorogenic substrate, which was capable of statistically differentiating UFH from 0 to 0.4 U/mL every 0.2 U/mL. Fig. 5 shows the normalized dose-response curve of UFH spiked whole blood donor samples. As can be seen, as the concentration of UFH increases, the log (dF/dt) decreases. The calibration curve plateaus beyond 0.4 U/mL UFH, rendering the assay insensitive beyond 0.4 U/mL (Table 1). %CV values were <10% up to 0.2 U/mL but increased to 15-25% between

0.4-0.8 U/mL. Linear regression analysis was performed within the UFH statistically sensitive range proving a linear calibration curve of $y = -1.87x + 1.70$ with an $R^2 > 0.99$.

4. Discussion

In the first part of this study, human pooled plasma samples were spiked with UFH concentrations from 0 to 1.6 U/mL every 0.2 U/mL. Two optimized assays covering the low (from 0-0.6 U/mL) and high range (from 0.6-1.2 U/mL) for UFH were developed. The fluorogenic anti-FXa assay concentrating on the low range of UFH was found to be 100 nM FXa and 25 μM SN-7 fluorogenic substrate which was capable of statistically differentiating the log of the slope values in commercial human pooled plasma from 0 to 0.6 U/mL every 0.2 U/mL. A linear calibration curve was calculated returning a correlation coefficient of $R^2 = 0.96$. Good reproducibility of results was achieved as indicated by CV values below 2%. In relation to the UFH high range concentrations, the optimized assay proved to be 200 nM FXa and 75 μM ANSN-based fluorogenic substrate. Assay sensitivity was statistically determined from 0.6 to 1.2 U/mL UFH with CV values less than 2%. The test revealed linearity within the statistically range with a correlation coefficient of $R^2 > 0.99$.

The low and high range fluorogenic anti-FXa assays developed for UFH compare positively with the different commercially available chromogenic anti-FXa assays in terms of assay sensitivity and reproducibility. Most of the tests from Chromogenix, American Diagnostica Inc., Hyphen Biomed and Instrumentation Laboratory to name just a few, measure therapeutic levels of UFH up to 1 U/mL with CV values between 5-10%. One of the limitations of the chromogenic anti-FXa assays is the lack of standardization as investigated by Ignjatovic et al. [25] and Kitchen et al. [26].

Another area of similar research is the development of fluorogenic thrombin generation assays in plasma [27] and whole blood [21, 28]. There are currently two commercially

available tests from Thrombinoscope and Technoclone, which use plasma, but lack of standardization is an issue [29, 30, 31]. Furthermore, it has been reported by Hemker et al. [22] that the relationship between thrombin generation and fluorescent activity is non-linear due to substrate consumption and the inner filter effect. In the low and high UFH range fluorogenic anti-FXa assays developed in the study presented here, linear calibration curves were calculated returning correlation coefficients of 0.96 and >0.99, respectively.

In the second part of the study, whole blood experiments were performed. Firstly, whole blood background fluorescence of the six donor samples was examined using the absorption and emission spectra. The main absorption peaks appeared at ~ 280, 345, 415, 541, and 576 nm in all samples, with the maximal absorption band at 415 nm. These results agree well with the absorption bands reported by Chen et al. [32] and Li et al. [33]. According to Chen et al. [32], these absorption bands are similar to that of red blood cells with the exception of the absorption peak at 280 nm. The band at ~ 280 nm corresponds to tryptophan with emission at 340 nm [33]. The bands at ~ 345 nm, 415 nm, and 576 nm have been correlated with the absorption of iron porphyrin, which is part of haemoglobin [34]. And the last two absorption peaks at ~541 nm and ~576 nm correspond to oxyhemoglobin [35]. Emission spectra of the six volunteers at 352 nm excitation wavelength, showed a very low fluorescence intensity emission peak at ca. 470 nm. Therefore, this emission band indicates the presence of an endogenous fluorophore in whole blood with similar excitation-emission wavelength pair to that of the ANSN fluorophore. The wavelength pair at ca. 340-460 nm has been attributed to endogenous reduced nicotinamide adenine dinucleotide or reduced nicotinamide adenine dinucleotide phosphate [33, 36]. Nevertheless, this fact does not represent an issue as the background fluorescence signal is only 1% of the total fluorescence range of the instrument.

Secondly, the fluorogenic assay was re-optimized in whole blood because of the small increment in fluorescence signal obtained compared with the results in plasma. Considering

that whole blood contains several components, the fluorescence signal could be diminished by light scattering effects as suggested Kim et al. [35]. Thus the optimized fluorogenic anti-FXa assay in whole blood was found to be 350 nM FXa and 87.5 μM SN-7 fluorogenic substrate, which was capable of statistically differentiating UFH from 0 to 0.4 U/mL every 0.2 U/mL. Linear regression of the statistically different data revealed good linearity ($y = -1.87x + 1.70$ and an $R^2 > 0.99$).

Several attempts have been made to develop sensitive and rapid UFH whole blood assays for critical clinical applications (e.g. cardiopulmonary bypass surgery, liver surgery, intensive care units, etc.) but no success has been achieved to date. ACT whole blood point-of-care devices are still widely used despite the drawbacks in relation to precision and sensitivity to low UFH concentrations [37, 38]. It has been reported that the detection limit for heparin in the ACT is ca. 0.5 U/mL therefore not being able to quantify normal therapeutic UFH concentrations compared to anti-FXa assays [39].

Murray et al. [30] compared the sensitivity of ACT, APTT, protamine titration, thromboelastography (TEG) and chromogenic anti-FXa assays to heparin therapy, in vascular and cardiac surgical patients. ACT was reported to be the least sensitive test to identify residual heparin. In another study, the new thrombolytic assessment system heparin management test (TAS HMT) was compared with ACT and a chromogenic anti-FXa assay as the reference method [37]. They documented that the new TAS HMT did not improve the performance of ACT. In addition, two fluorogenic thrombin generation (TG) assays have been developed in whole blood but not tested in the presence of UFH [21, 28]. Tappenden et al. [28] showed that the whole blood TG assay was more sensitive than the PPP or PRP methods, but its imprecision was higher with CV values for peak height and endogenous thrombin potential of 14% and 13%, respectively. In our case, CVs were also higher in whole blood than in plasma samples at all concentrations with values between 4-20%.

And finally, considering that FXa is the method of reference for measuring UFH, Hansen et al. [40] developed an ACT II anti-Xa assay in whole blood with in vitro experiments revealing linearity up to 6 U/mL UFH. Despite its rapidity and ease-of-use, individual calibration curves need to be carried out in the operating room, which could hamper assay procedure.

5. Conclusions

Apart from two recent publications on the development of fluorogenic anti-FXa assays to monitor anticoagulant therapy in human pooled plasma [23, 24], to the best of our knowledge, no studies on whole blood have been previously described. This paper outlines the development of two more fluorogenic anti-FXa assays using one of the only two commercially available FXa fluorogenic substrates. Two assays were developed to measure low (0-0.6 U/mL) and high (0.6-1.2 U/mL) ranges of UFH in human pooled plasma. Moreover, a linear calibration curve up to 0.4 U/mL UFH using the fluorogenic anti-FXa assay in whole blood was developed. Further studies need to be undertaken to evaluate the potential of this latter novel fluorogenic anti-FXa assay in critical clinical situations where monitoring low UFH concentrations is needed.

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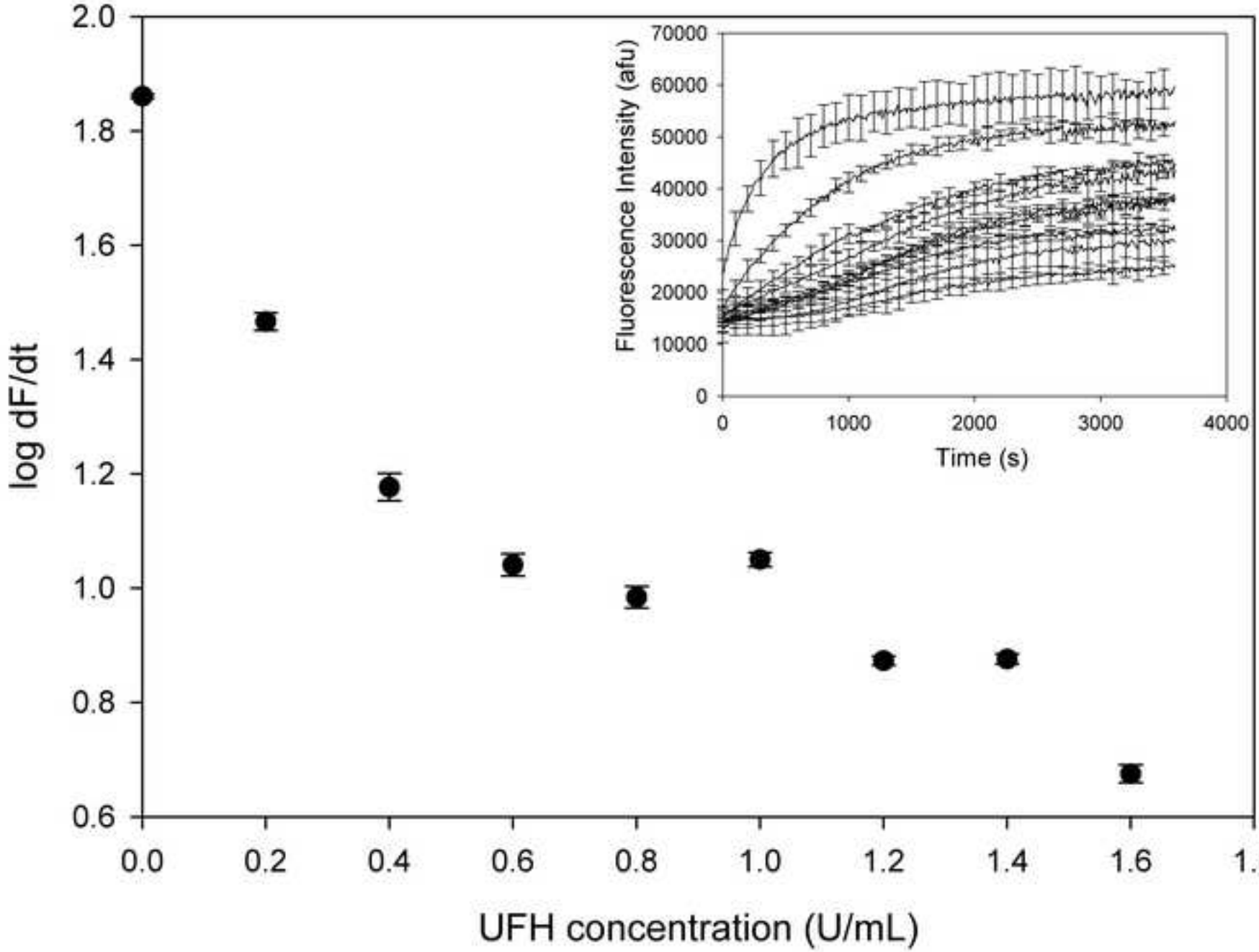
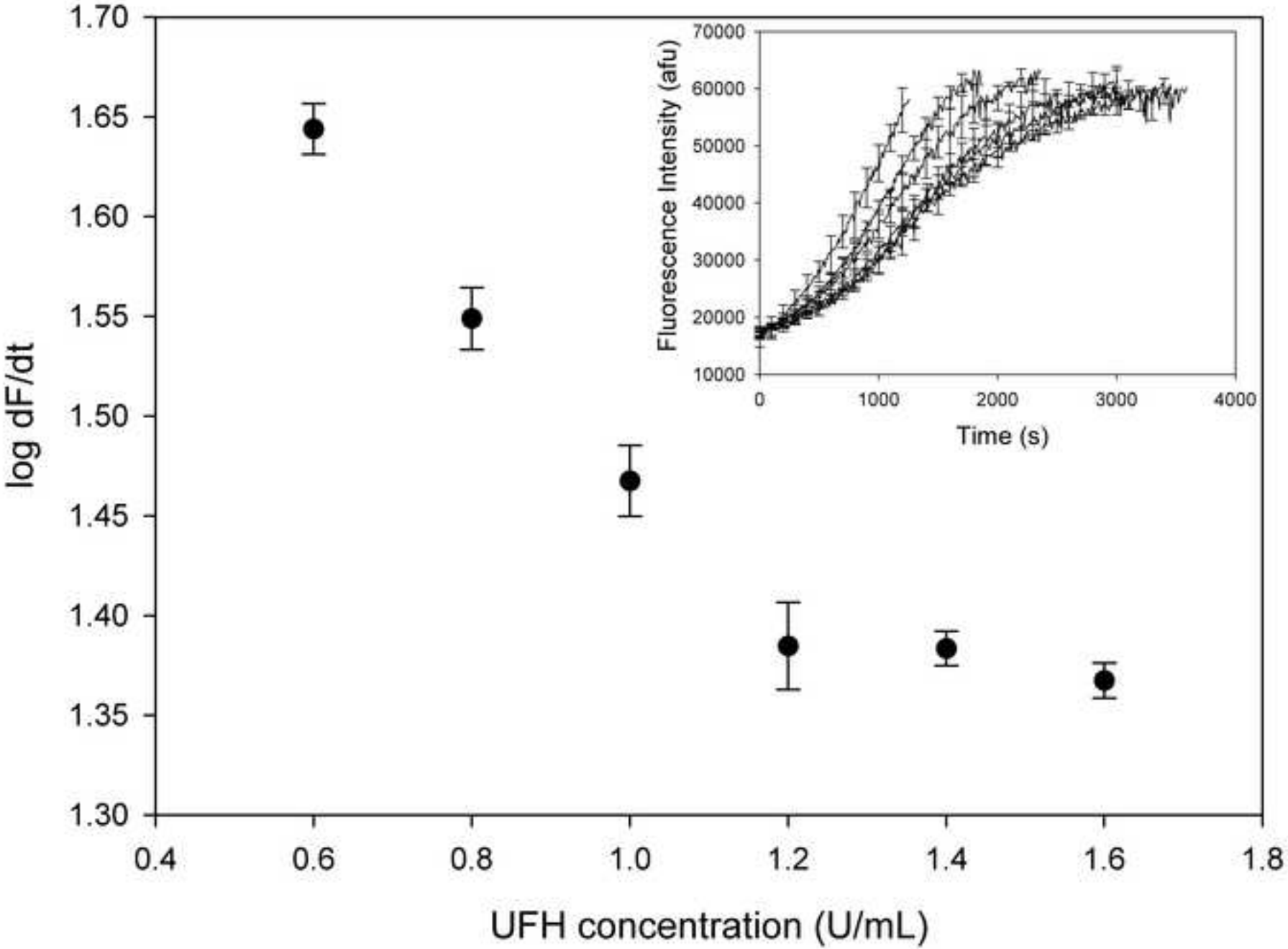
Table 1. Summary of averaged log_{10}-transformation of reaction rates, SD and %CV values of the fluorogenic anti-FXa assay within the low UFH range of 0-0.8 U/mL, high UFH range of 0.6-1.6 U/mL in plasma (n=3), and whole blood (n=6).

	Plasma			**Whole blood**		
[UFH] (U/mL)	**Low UFH range**					
	log(dF/dt)	**SD**	**CV (%)**	**log(dF/dt)**	**SD**	**CV (%)**
0	1.86	0.00	0.20	1.70	0.07	4.00
0.2	1.47	0.02	1.04	1.32	0.09	6.72
0.4	1.18	0.02	2.04	0.95	0.19	20.04
0.6	1.04	0.02	1.87	0.89	0.21	23.32
0.8	0.98	0.02	1.93	0.90	0.14	15.41
	High UFH range					
0.6	1.64	0.01	0.77			
0.8	1.55	0.02	1.00			
1	1.47	0.02	1.21			
1.2	1.38	0.02	1.58			
1.4	1.38	0.01	0.62			
1.6	1.37	0.01	0.65			

Figure(s)


log dF/dt
UFH concentration (U/mL)
Fluorescence Intensity (afu)
Time (s)

Figure(s)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1.65
1.60
1.55
1.50
1.45
1.40
1.35
1.30
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
UFH concentration (U/mL)
Fluorescence Intensity (afu)
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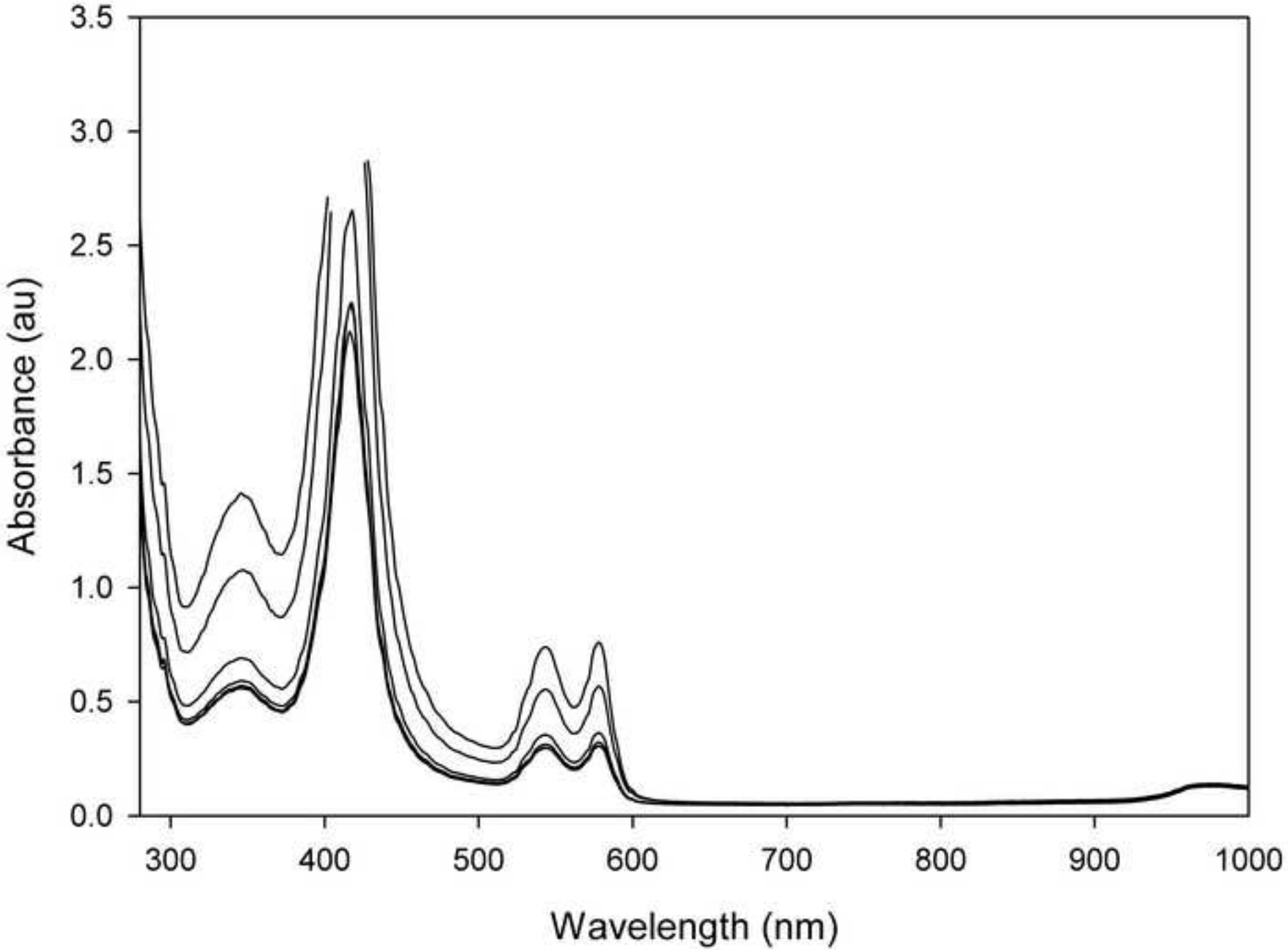
60000
50000
40000
30000
20000
10000
0
1000
2000
3000
4000
Time (s)

Figure(s)


Absorbance (au)
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0.5
1.0
1.5
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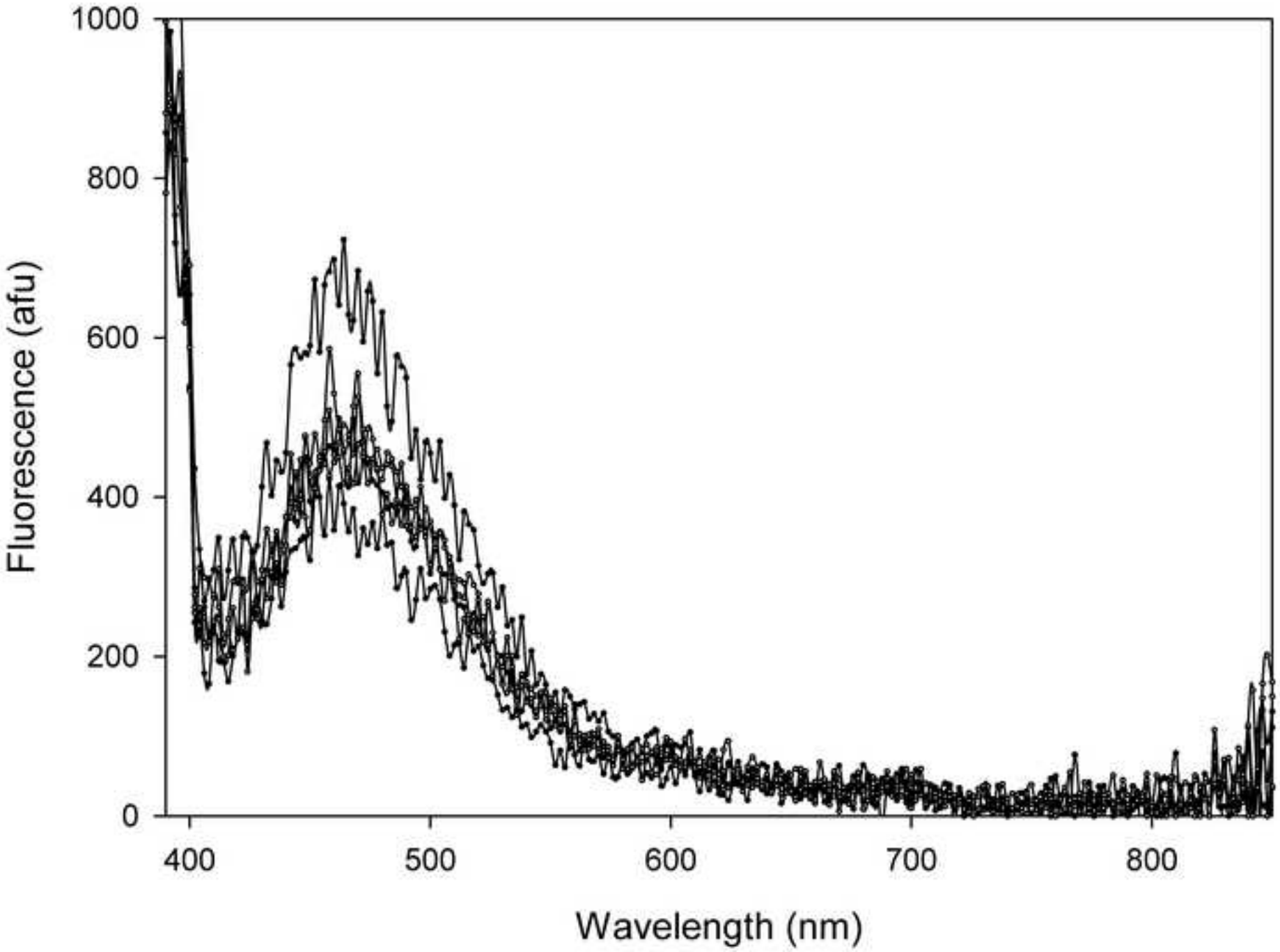
3.5
300
400
500
600
700
800
900
1000
Wavelength (nm)

Figure(s)


1000
800
600
400
200
0
Fluorescence (afu)
400
500
600
700
800
Wavelength (nm)

Figure(s)

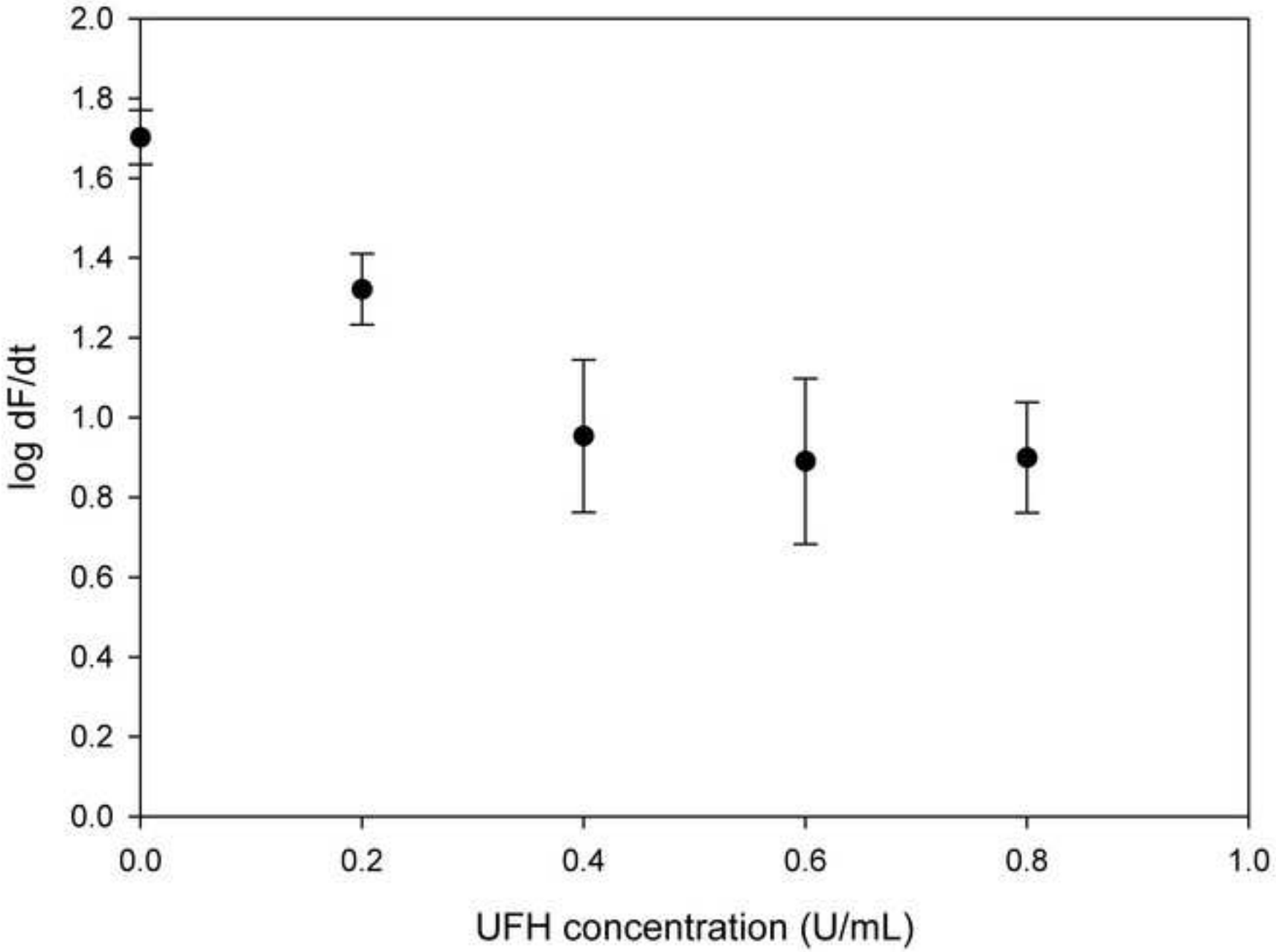

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
log dF/dt
0.0
0.2
0.4
0.6
0.8
1.0
UFH concentration (U/mL)

Figure Captions

Fig. 1. Log/lin dose-response curve of UFH within the range of 0–1.6 U/ml in human pooled plasma. Inset: Reaction progress curves of the ANSN-based fluorogenic anti-FXa assay (100 nM FXa and 25 μM ANSN fluorogenic substrate) using human pooled plasma spiked with UFH (0-1.6 U/ml) (n=3).

Fig. 2. Log/lin dose-response curve of UFH within the range of 0.6–1.6 U/ml in human pooled plasma. Inset: Reaction progress curve of the ANSN-based fluorogenic anti-FXa assay (200 nM FXa and 75 μM ANSN fluorogenic substrate) using human pooled plasma spiked with UFH (0.6-1.6 U/ml) (n=3).

Fig. 3. Absorption spectra of whole blood from six donors evaluated from 280 to 1000 nm.

Fig. 4. Fluorescence emission spectra (400 – 850 nm) of whole blood from six donors at $\lambda_{excitation}$= 352 nm.

Fig. 5. Log/lin dose-response calibration curve of UFH within the range of 0-0.8 U/ml in whole blood samples (n=6).