2010

Comparative Study of Factor Xa Fluorogenic Substrates and Their Influence on the Quantification of LMWHs

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Recommended Citation

Castro-Lopez, V., Harris, L., O'Donnell, J. and Killard, A. (2011). Comparative study of Factor Xa fluorogenic substrates and their influence on the quantification of LMWHs. ,*Analytical and Bioanalytical Chemistry*, 2011 Jan;399(2):691-700. doi:10.1007/s00216-010-4314-3

Funder: Enterprise Ireland

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1	Article Title	**Comparative study of Factor Xa fluorogenic substrates and their influence on the quantification of LMWHs**	
2	Article Sub- Title		
3	Article Copyright - Year	**Springer-Verlag 2010 (This will be the copyright line in the final PDF)**	
4	Journal Name	Analytical and Bioanalytical Chemistry	
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40		Received	18 August 2010
41	Schedule	Revised	6 October 2010
42		Accepted	7 October 2010

43	Abstract	Low molecular weight heparins (LMWHs) are recognised as the preferred anticoagulants in the prevention and treatment of venous thromboembolism. Anti-Factor Xa (anti-FXa) levels are used to monitor the anticoagulant effect of LMWHs and such assays are routinely employed in hospital diagnostic laboratories. In this study, a fluorogenic anti-FXa assay was developed using a commercially available fluorogenic substrate with an attached 6-amino-1-naphthalene-sulfonamide (ANSN) fluorophore and was used for the determination of two LMWHs, enoxaparin and tinzaparin and the heparinoid, danaparoid. The assay was based on the complexation of heparinised plasma with 100 nM exogenous FXa and 25 μM of the fluorogenic substrate Mes-D-LGR-ANSN $(C_2H_5)_2$ (SN-7). The assay was tested with pooled plasma samples spiked with anticoagulant concentrations in the range 0–1.6 U mL^{-1}. The statistically sensitive assay range was 0–0.4 U mL^{-1} for enoxaparin and tinzaparin and 0–0.2 U mL^{-1} for danaparoid, with assay variation typically below 10.5%. This assay was then compared with a previously published fluorogenic anti-FXa assay developed with the peptide substrate, methylsulfonyl-d-cyclohexylalanyl-glycyl-arginine-7-amino-4-methylcoumarin acetate (Pefafluor FXa). Both assays were compared in terms of fluorescence intensity, lag times and sensitivity to anticoagulants.

44	Keywords separated by ' - '	FXa - Fluorogenic substrate - AMC - ANSN - Low molecular weight heparins - Danaparoid
45	Foot note information	This paper was published in the special issue *Heparin Characterization* with Guest Editor Cynthia K. Larive.

Anal Bioanal Chem
DOI 10.1007/s00216-010-4314-3

ORIGINAL PAPER

Comparative study of Factor Xa fluorogenic substrates and their influence on the quantification of LMWHs

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Received: 18 August 2010 / Revised: 6 October 2010 / Accepted: 7 October 2010

Abstract Low molecular weight heparins (LMWHs) are recognised as the preferred anticoagulants in the prevention and treatment of venous thromboembolism. Anti-Factor Xa (anti-FXa) levels are used to monitor the anticoagulant effect of LMWHs and such assays are routinely employed in hospital diagnostic laboratories. In this study, a fluorogenic anti-FXa assay was developed using a commercially available fluorogenic substrate with an attached 6-amino-1-naphthalene-sulfonamide (ANSN) fluorophore and was used for the determination of two LMWHs, enoxaparin and tinzaparin and the heparinoid, danaparoid. The assay was based on the complexation of heparinised plasma with 100 nM exogenous FXa and 25 μM of the fluorogenic substrate Mes-D-LGR-ANSN $(C_2H_5)_2$ (SN-7). The assay was tested with pooled plasma samples spiked with anticoagulant concentrations in the range 0–1.6 U mL^{-1}. The statistically sensitive assay range was 0–0.4 U mL^{-1} for enoxaparin and tinzaparin and 0–0.2 U mL^{-1} for danaparoid, with assay variation typically below 10.5%. This assay was then compared with a previously published fluorogenic anti-FXa assay developed with the peptide substrate, methylsulfonyl-D-cyclohexylalanyl-glycyl-arginine-7-amino-4-methylcoumarin acetate (Pefafluor FXa). Both assays were compared in terms of fluorescence intensity, lag times and sensitivity to anticoagulants.

Keywords FXa · Fluorogenic substrate · AMC · ANSN · Low molecular weight heparins · Danaparoid

Introduction

Low molecular weight heparins (LMWHs) are efficacious anticoagulants administered for both the prophylaxis and treatment of venous and arterial thromboembolic disorders and acute coronary syndromes [1–3]. LMWHs require less laboratory monitoring than unfractionated heparin (UFH) as they exhibit a more predictable pharmacokinetic response with reduced bleeding risk [4]. However, clinical situations do arise where the anticoagulant effect of LMWHs is unpredictable. As a result, laboratory monitoring of LMWHs is necessary in special patient cohorts including pregnant women, the elderly, children, patients with renal insufficiency, and patients at the extremes of body weight [2, 5].

The recommended test for monitoring LMWHs therapy is the anti-Factor Xa (anti-FXa) assay due to the strong inhibition of FXa by LMWHs [5]. The first anti-FXa assay was a clotting time test based on the heparin-accelerated inhibition of FXa [6]. Anti-FXa assays currently employed in the central laboratory setting are chromogenic and use a synthetic FXa peptide substrate coupled to a cleavable chromophore [7] and [2]. The application of synthetic peptide substrates to the analysis of coagulation proteins confers many advantages over traditional clot-based assays, in terms of greater sensitivity, specificity, accuracy, and simplicity [8].

This paper was published in the special issue *Heparin Characterization* with Guest Editor Cynthia K. Larive.

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The endopeptidases involved in coagulation and fibrinolysis are trypsin-like serine endopeptidases [9]. Research into the use of synthetic substrates for the evaluation of coagulation endopeptidases began in the 1950s and 1960s. Initially, chromogenic substrates based on the chromophore *p*-nitroaniline were developed for thrombin, plasmin, and trypsin analysis [8, 10], followed by the development of assays using fluorogenic substrates for thrombin [11, 12]. Once the specific peptide cleavage sequence was known, these developments were rapidly extended to other coagulation proteins in the 70s and 80s, resulting in a new strategy for the biochemical study of the coagulation system [10].

Factor Xa occupies a critical position in the coagulation cascade; hence its joint popularity with thrombin as a therapeutic target for anticoagulant therapy. The first photometric anti-FXa assay was introduced in 1976 [13] followed by the rapid development of more specific chromogenic substrates and eventually a fluorogenic substrate specific for FXa which was introduced in 1977 [14]. Both chromogenic and fluorogenic assays are well suited to the measurement of heparin, as there is a direct relationship between the functional activity of heparin and its effect on antithrombin allowing for greater precision in its determination [8]. Fluorescence-based measurements offer further advantages over colorimetric assays, such as superior sensitivity and specificity [14, 15], as well as measurement in a broader range of sample types such as platelet poor plasma, platelet rich plasma, and whole blood [16, 13].

Two fluorophores are principally used in commercial substrates for the analysis of coagulation proteins, namely 7-amino-4-methylcoumarin (AMC) and 6-amino-1-naphthalene-sulfonamide (ANSN) [17, 18, 19, 20]. The majority of studies have focussed on the reactivity of substrates containing 7-amino-4-methylcoumarin, as it is the most common fluorogenic leaving group used in bioassays [21]. Coumarins can be described as a broad class of fluorophores resulting in UV or near-UV excitation wavelengths with a high quantum yield and low extinction coefficient [22]. The naphthalenesulfonamides were reported in 1992 as a new fluorescent group for substrates of amidases which are also excited in the UV range. Their high quantum yield and the presence of a sulfonyl moiety allows for a range of chemical modifications which can enhance enzymatic substrate specificity [9]. However, given the availability of these substrates, few publications have evaluated their usefulness in the development of anti-FXa assays.

A novel fluorogenic anti-FXa assay was developed in this study using a peptide substrate for FXa with an attached ANSN fluorophore and was compared to a previously developed assay based on an AMC fluorescent leaving group [16]. A comparison of assay responses to pharmacological concentrations of LMWHs and danaparoid in commercial pooled plasma is reported.

Materials and methods

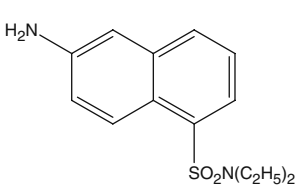
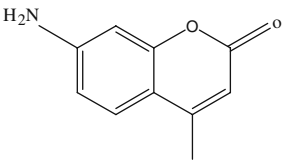
Reagents

Water (ACS 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 $CaCl_2$ 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 (Pefafluor 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 were prepared in 10 mM HEPES. Tubes were covered with aluminium foil to protect from exposure to light. The fluorogenic substrate Mes-D-LGR-ANSN $(C_2H_5)_2$ (SN-7), containing the fluorescent reporter group 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 aluminium foil. Dilutions of stock solutions were performed with 10 mM HEPES. Table 1 summarises the kinetic constants and physico-chemical properties of both the Pefafluor FXa and SN-7 fluorogenic substrates. These parameters were obtained from the suppliers. Kinetic constants refer to the cleavage of both fluorogenic substrates by the endopeptidase FXa. Purified human FXa (serine endopeptidase; code number: EC 3.4.21.6) was obtained from HYPHEN BioMed (Neuville-Sur-Oise, France). Tinzaparin (Innohep®) was obtained from LEO Pharma (Ballerup, Denmark). Enoxaparin (Clexane®) and danaparoid (Orgaran®) were from 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 stabilise for at least 20 min at room temperature prior to use.

Apparatus and software

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™ micro-

t1.1 **Table 1** Kinetic constants and physico-chemical properties of both fluorogenic substrates

Fluorogenic substrate	**SN-7**	**Pefafluor FXa**
Formula	Mes-D-LGR-ANSN(C_2H_5)$_2$	CH_3SO_2-D-CHA-Gly-Arg-AMC.AcOH
Physical form	Liquid	Lyophilised powder
Fluorophore chemical structure		
M_w (g mol^{-1})	682.8	679.8
K_m (μM)	125	220
k_{cat} (s^{-1})	36	162
k_{cat}/K_m (M^{-1} s^{-1})	290,000	~740,000
Wavelength maxima (nm)	352 (λ_{ex}), 470 (λ_{em})	342 (λ_{ex}), 440 (λ_{em})

plates from Thermo Fisher Scientific (Roskilde, Denmark) were used.

Fluorogenic anti-FXa assay

Measurements were carried out in reconstituted citrated human pooled plasma. FXa and the ANSN-based fluorogenic substrate were titrated within the range of 0.1–100 nM and 8.3–33.3 μM (K_m=125 μM), respectively. Samples consisting of 6.25 μL of 100 mM $CaCl_2$, 43.75 μL of pooled plasma, and 50 μL of FXa (0.1–100 nM) 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 (8.3–33.3 μM). 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 integration time of 20 μs. 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 samples were spiked with pharmacologically relevant concentrations (0–1.6 U mL^{-1}) of therapeutic anticoagulants including enoxaparin, tinzaparin, and danaparoid. 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. The assay using the Pefafluor FXa fluorogenic substrate was optimised as previously described by Harris et al. [16]. The fluorescence excitation and emission wavelengths were 342 nm and 440 nm, respectively, corresponding to the excitation/emission wavelengths of the AMC fluorophore. The only difference between these assays was the insertion of an incubation step in the ANSN-based assay to improve reproducibility.

Statistical analysis

All graphs were plotted using SigmaPlot 8.0. and SPSS 17.0 was used for statistical analysis. Intra-assay differences within the anticoagulant concentration range were compared using one-way analysis of variance (ANOVA), with subsequent post-hoc analysis performed (Scheffe's test) if significance was observed. A result of $p<0.05$ was considered statistically significant. Inter-assay differences between the two fluorogenic substrates employed were statistically analysed using the paired Student's t test at a significance level of 5%.

Results

Assay optimisation

In this study, two fluorogenic substrates with two different fluorophore leaving groups were compared using the anti-FXa assay principle, whereby exogenous FXa added to heparinised plasma was inhibited by the heparin–AT complex and the resulting FXa activity was measured using fluorescence. A new assay is reported that measures the rate

of ANSN fluorophore release as a result of FXa substrate cleavage and is compared to the rate of AMC fluorophore release which has been published previously [16]. Optimised assay concentrations for this new fluorogenic assay were determined by performing titrations of FXa from 0.1 to 100 nM and fluorogenic substrate concentrations from 8.3 to 33.3 μM. To allow for differentiation in the assay reaction rates, the concentration range of the ANSN-based substrate was selected so that it was lower than the K_m value. The final working assay concentrations of 25 μM ANSN-based fluorogenic substrate and 100 nM FXa, conform to the optimisation criteria which included short lag times, fast reaction rates, and a broad fluorescence dynamic range for the differentiation of anticoagulant concentrations. The same criteria were also selected for the optimization of the AMC-based fluorogenic anti-FXa assay.

Evaluation of the ANSN-based anti-FXa fluorogenic assay

Three anticoagulant drugs were tested using the fluorogenic anti-FXa assay with the ANSN-based substrate in commercial pooled plasma.

The fluorescence intensity profiles over time for tinzaparin plasma samples in the fluorogenic anti-FXa assay can be seen in Fig. 1 and the inset shows the dose–response profile versus anticoagulant concentration. As the concentration of tinzaparin increased, the fluorescence profiles reached lower fluorescence intensity values after 1 h. In the absence of tinzaparin, the maximum intensity value was 56,000 AU; with 0.2 U mL^{-1} the profile reached 40,000 AU and 27,000 AU for 0.4 U mL^{-1}. From 0.4 to 1.6 U mL^{-1}, the maximum fluorescence intensity value was between 22,000 and 27,000 AU.

Lag times were calculated by extrapolation of the linear portion of the progression curve to its intersection with the x-axis [23]. In the presence of higher anticoagulant concentrations, longer lag time values were observed. At low concentrations of 0 and 0.2 U mL^{-1} tinzaparin, the reaction was very quick and no lag time was observed. Lag times increased to 310 and 630 s for 0.4 U mL^{-1} and 0.6 U mL^{-1} respectively. At concentrations of 0.8–1.6 U mL^{-1} lag time values were 1,010 s.

The reaction rates of the fluorescence profiles were calculated to generate dose–response profiles and a similar trend was observed for all drugs analysed, in that as anticoagulant concentration increased, the rate of the reaction decreased. The slope values were statistically different up to 0.4 U mL^{-1} ($p<0.003$) with significantly reduced sensitivity in the upper tinzaparin range (0.6–1.6 U mL^{-1}).

The anti-FXa assay was also tested with enoxaparin, another member of the low molecular weight heparin family. The fluorescence profiles are very similar to those of tinzaparin (Fig. 2). The fluorescence profiles for 0 U mL^{-1} reached 52,000 AU after 1 h, for 0.2 U mL^{-1} the intensity value was 38,000 AU and with 0.4 U mL^{-1} the profile reached 28,000 AU. At higher enoxaparin concentrations, the profiles reached maximum intensity values of 21,000 and 28,000 AU.

At low concentrations of enoxaparin lag times were absent but were calculated as 110 s for 0.4 and 0.6 U mL^{-1} enoxaparin. At higher concentrations, lag times increased to 590 s for 0.8 and 1 U mL^{-1}, 730 s for 1.2 U mL^{-1}, and 910 s for 1.4 and 1.6 U mL^{-1}.

The dose–response curve calculated from the linear slopes of the fluorescence profiles of enoxaparin can be

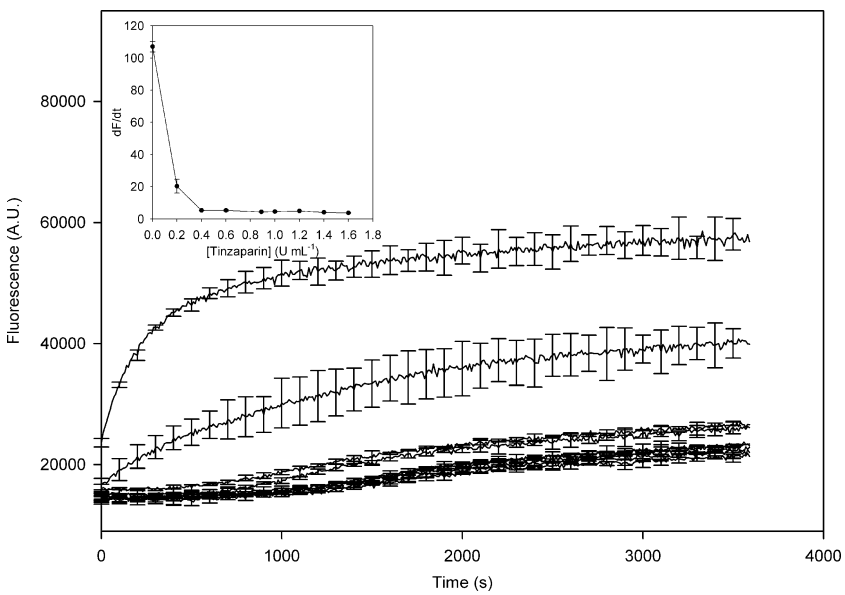


Fig. 1 Fluorescence intensity vs. time for the anti-FXa activity with tinzaparin (n=3). Tinzaparin concentration increases from top to bottom. *Inset* Dose–response calibration curve of tinzaparin in pooled plasma in the ANSN-based fluorogenic anti-FXa assay (n=3), calculated from the linear slopes (dF/dt) of the fluorescence response curves

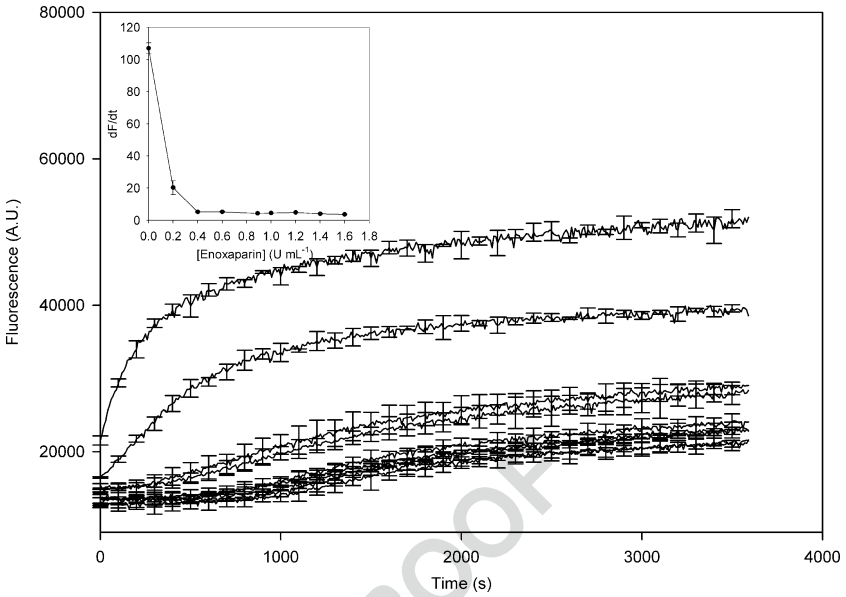


Fig. 2 Fluorescence intensity vs. time for the anti-FXa activity with enoxaparin (n=3). Enoxaparin concentration increases from top to bottom. *Inset* Dose–response calibration curve of enoxaparin in pooled plasma in the ANSN-based fluorogenic anti-FXa assay (n=3), calculated from the linear slopes (dF/dt) of the fluorescence response curves

seen in Fig. 2 and good sensitivity was achieved with concentrations up to 0.4 U mL^{-1} (p<0.003).

Figure 3 shows the fluorescence profiles of the anti-Xa assay in the presence of danaparoid. The fluorescence profile for 0 U mL^{-1} reached 55,000 AU after 1 h, at 0.2 U mL^{-1} the maximum fluorescence intensity value was 35,000 AU and from 0.4 to 1.6 U mL^{-1} danaparoid, profiles reached fluorescence intensities between 22,000 and 29,000 AU.

Similar to the LMWHs, lag times were not observed at low concentrations of danaparoid. Lag times increased from 200 s at 0.4 U mL^{-1} to 780 s at 0.6 U mL^{-1}, 1,050 s at 0.8 U mL^{-1} and 1180 s at 1 U mL^{-1}. At 1.2 U mL^{-1} and 1.6 U mL^{-1} danaparoid lag times were 1,320 s and at 1.4 U mL^{-1} the lag time was calculated as 1,480 s.

Inset of Fig. 3 shows the dose–response calibration curve of danaparoid in the fluorogenic anti-FXa assay. Statistical analysis returned significant differences in the variances with the Levene's test (p=0.011), so one-way ANOVA could not be applied. When equal variances were not assumed, the Tamhane test returned significant differences between 0 and 0.2 U mL^{-1} danaparoid (p=0.031).

The reaction rates were compared for all drugs tested using the normalised data (ratios of the dose–responses against the averaged dose–response value at 0 U mL^{-1}). The reaction rates for enoxaparin were higher than tinzaparin rates at all concentrations from 0 to 1.6 U mL^{-1}. Both low molecular weight heparins returned higher reaction rates than danaparoid in the statistically sensitive range of 0–0.2 U mL^{-1}. Enoxaparin reaction rates were the highest of

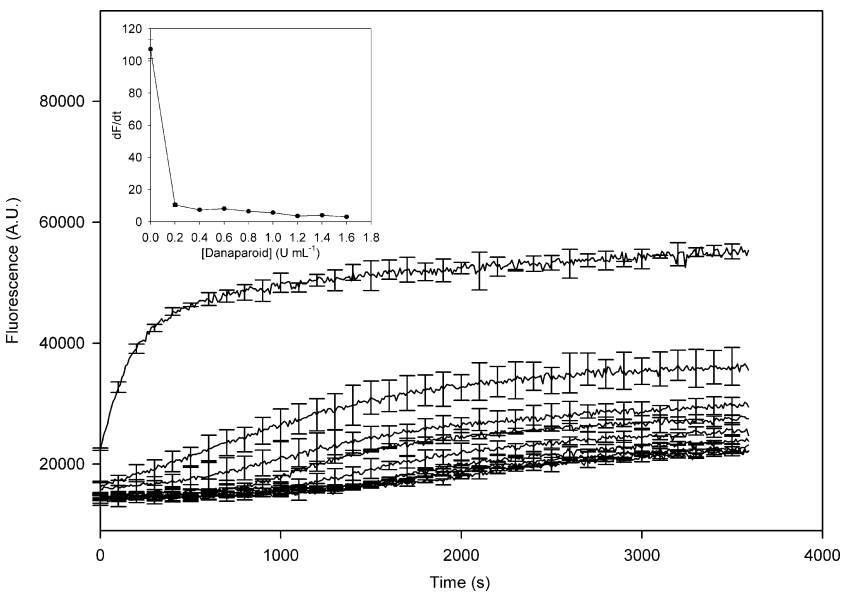


Fig. 3 Fluorescence intensity vs. time for the anti-FXa activity with danaparoid (n=3). Danaparoid concentration increases from top to bottom. *Inset* Dose–response calibration curve of danaparoid in pooled plasma in the ANSN-based fluorogenic anti-FXa assay (n=3), calculated from the linear slopes (dF/dt) of the fluorescence response curves

JnlID 216_ArtID 4314_Proof# 1 - 14/10/2010

all drugs tested and only at concentrations of 0.6, 0.8, and 1 U mL^{-1}, were tinzaparin reaction rates lower than danaparoid, but these fall into the statistically insensitive range.

The analytical errors (standard deviation and percentage coefficient of variation) related to each of the slope measurements for all three anticoagulants were calculated. All CV values were <10.5% with the exception of 0.2 U mL^{-1} tinzaparin which returned a CV value of 21%.

Comparative studies of AMC versus ANSN-based fluorogenic assays

The two synthetic peptide substrates containing two different fluorogenic leaving groups were compared in terms of their fluorescence intensity, lag times, and slope values.

Fluorescence intensity

All three of the AMC-based fluorogenic assay reaction progress curves for enoxaparin, tinzaparin, and danaparoid, reported by Harris et al. [16], approached a similar maximum plateau value of product formation at approximately the same level (i.e., 48,000–53,000 AU) independent of anticoagulant type and concentration. This is an indication of substrate depletion and also that the enzyme remains stable under the conditions tested. In the ANSN-based study, the maximum value of product formed for all three anticoagulant drugs reached different fluorescence intensity values depending on anticoagulant concentration. As the concentration of anticoagulant increased from 0 to 1.6 U mL^{-1}, the fluorescence intensity values decreased from 56,000 to 22,000 for tinzaparin, 52,000 to 21,000 for enoxaparin and finally, from 55,000 to 22,000 for danaparoid.

Lag time values

The increase in lag time with increasing anticoagulant concentration was common to both fluorogenic assays as shown in Table 2. Furthermore, the ANSN-based anti-FXa fluorogenic assay returned smaller lag time values than the AMC-based assay for all anticoagulant concentrations.

Slope values

Analysis of slope values at 0 U mL^{-1} returned 25% variation in all assays. As a result, all slope values were normalised with respect to those at 0 U mL^{-1} for each anticoagulant. As can be seen in Fig. 4, the fluorogenic anti-FXa assay using the AMC fluorogenic substrate returned higher slopes than the ANSN fluorogenic assay in all instances. The inter-assay variability between the two fluorogenic substrates for each anticoagulant drug was statistically evaluated using the paired Student's *t* test. Results indicate that there is a significant mean difference ($p<0.05$) between the two fluorogenic anti-FXa assays for each anticoagulant at all concentrations, except for 0 U mL^{-1} as it corresponds to the unit value of normalisation in all cases.

Assay sensitivity

The intra-assay variability between different concentrations of anticoagulants was assessed for both fluorogenic substrates. Table 3 outlines the statistically sensitive range for both fluorogenic anti-FXa assays each containing a different leaving group in the presence of tinzaparin, enoxaparin, and danaparoid. When the ANSN-based fluorogenic anti-FXa assay was performed in the presence of tinzaparin, the slopes of the enzymatic reaction were statistically different up to 0.4 U mL^{-1} ($p<0.05$) at intervals of 0.2 U mL^{-1}. In the case of the Pefafluor FXa substrate the assay proved to be sensitive up to 0.6 U mL^{-1} tinzaparin ($p<0.001$). This reduction in assay sensitivity for the ANSN-based fluorogenic anti-FXa assay was also observed for enoxaparin. The fluorogenic anti-FXa assay using the ANSN-based substrate resulted in a narrower statistically sensitive range from 0 to 0.4 U mL^{-1} ($p<0.05$) when compared with the AMC-based substrate assay range of 0–0.8 U mL^{-1} ($p<0.001$) for enoxaparin. The effect of danaparoid was also evaluated in both fluorogenic assays and a larger difference in assay sensitivity was observed. Response slopes to danaparoid concentrations at 0.2 U mL^{-1} intervals were statistically different up to 0.2 U mL^{-1} and 1 U mL^{-1} for the ANSN and AMC-based substrate assays, respectively. In all cases, the AMC-based substrate seems to offer a wider assay sensitive range compared with the ANSN-based fluorogenic substrate when quantifying both LMWHs and danaparoid.

Reproducibility (%CV values)

Reproducibility of the two developed assays was determined using the percentage coefficient of variation (%CV). Comparing CV values between the two fluorogenic assays, results in lower variation for both low molecular weight heparins and danaparoid with the AMC fluorophore. Studies of precision for the fluorogenic anti-FXa assay using the AMC-based substrate showed a coefficient of variation ranging between 0.5% and 7% [16]. In the case of the ANSN-based assay, CV values were below 10.5% except for 0.2 U mL^{-1} tinzaparin which returned a CV of 21%.

t2.1 **Table 2** Lag times of averaged progress curves for both anti-FXa fluorogenic assays in the presence and absence of tinzaparin, enoxaparin, and danaparoid

Conc (U mL^{-1})	Enoxaparin[a] (s)		Tinzaparin[a] (s)		Danaparoid[a] (s)	
	AMC	ANSN	AMC	ANSN	AMC	ANSN
0	200	0	160	0	220	0
0.2	280	0	240	0	560	0
0.4	420	110	380	310	800	200
0.6	580	110	670	630	1,420	780
0.8	850	590	900	1,010	2,010	1,050
1	940	590	1,040	1,010	2,070	1,180
1.2	1,020	730	1,440	1,010	2,440	1,320
1.4	1,260	910	1,800	1,010	>3,600	1,480
1.6	1,310	910	2,000	1,010	>3,600	1,320

[a] Averaged of triplicate measurements (n=3)

Discussion

FXa is a serine endopeptidase which occupies a central role in the coagulation cascade. LMWHs are anticoagulant drugs used in the treatment of thrombosis and cardiovascular diseases and can effectively neutralise FXa to prevent bleeding [24]. At present, commercially available anti-FXa tests capable of measuring LMWHs within the therapeutic range are all chromogenic assays. While chromogenic anti-FXa assays have some advantages over traditional clot-based assays, they do still suffer from some drawbacks as highlighted by Harris et al. [16]. To counteract these limitations, they presented a new fluorogenic anti-FXa assay based on the AMC fluorogenic substrate which can differentiate and quantify UFH, enoxaparin, tinzaparin, and danaparoid within the therapeutic range and with CV values below 7%.

A number of studies have been devoted to the identification of specific FXa fluorogenic substrates with desirable properties, such as the photo and chemical stability of the generated fluorescent group, the Stokes shift, the solubility of substrate and fluorescent product in aqueous buffer, and the fluorescence quantum yield, to mention just a few [9]. To date, several synthetic fluorogenic substrates targeting FXa have been developed with different peptidic groups, attached to just two fluorescent detecting groups namely ANSN [9] and AMC [14].

This paper sought to develop a new fluorogenic anti-FXa assay using the commercially available ANSN-based fluorogenic substrate for FXa and compare it with the AMC-based fluorogenic anti-FXa assay previously investigated by Harris et al. [16] in the presence and absence of two LMWHs (i.e., enoxaparin and tinzaparin) and danaparoid.

The principle of the assay is to measure the rate of fluorescent tag release which results from FXa proteolysis of the two synthetic peptide substrates, in the presence and absence of three anticoagulants. From the results obtained, different observations can be made: firstly, the anti-FXa

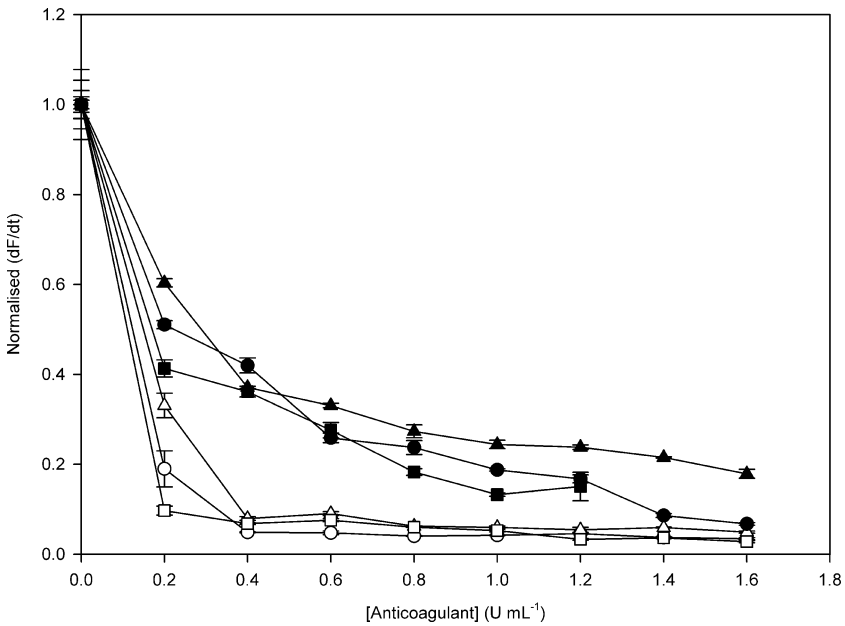


Fig. 4 Dose–response curves (normalised data) of the fluorogenic: ANSN-based anti-FXa assay to tinzaparin (*empty circle*), enoxaparin (*empty upright triangle*), and danaparoid (*empty square*); AMC-based anti-FXa assay to tinzaparin (*filled circle*), enoxaparin (*filled upright triangle*), and danaparoid (*filled square*)

t3.1 **Table 3** Comparison of the statistically sensitive range for each anticoagulant tested in two fluorogenic anti-FXa assays based on different fluorophore leaving groups

t3.2 Anticoagulant drug	Statistically sensitive range (U mL^{-1})	
t3.3	ANSN	AMC
t3.4 Tinzaparin	0–0.4	0–0.6
t3.5 Enoxaparin	0–0.4	0–0.8
t3.6 Danaparoid	0–0.2	0–1

assay presented in this paper was statistically differentiated at intervals of 0.2 up to 0.4 U mL^{-1} for enoxaparin and tinzaparin. It was also found to be statistically sensitive for danaparoid up to 0.2 U mL^{-1}. CV values were <10.5% for all anticoagulant drugs except for tinzaparin at 0.2 U mL^{-1}, which returned 21%. The sensitivity and dynamic range of the fluorogenic anti-FXa assay developed in this study are smaller than the values shown by the commercially available chromogenic anti-FXa assays. Most of the chromogenic anti-FXa assays are able to measure concentrations of LMWHs up to 1 U/ml with %CV values between 5% and 10% [16].

Secondly, the reaction progress curves of the ANSN-based assay show a very different time-course of product formation compared with those of the AMC-based assay. In this study, we observed a decrease in fluorophore formation with increasing anticoagulant concentration. In other words, the reaction progress curves reach lower fluorescence intensities as the concentration of anticoagulant increases. This could be indicative of inhibition by the accumulating products, instability of the enzyme or some other component of the assay system, the presence of enzyme-activated irreversible inhibitors [25], i.e., antithrombin (ATIII) in human plasma or the anticoagulants added to the assay. In comparison, the AMC-based fluorogenic assay showed similar maximum plateau values at all concentrations of FXa inhibitors, indicating enzyme stability and fluorogenic substrate depletion. Moreover, the ANSN substrate did not yield as good a signal-to-noise ratio as the AMC-based substrate; however, the former was not consumed as rapidly.

Thirdly, lag time values for the ANSN-based assay were shorter compared with the AMC-based assay for all anticoagulants and at all concentrations. Considering that the K_m value for ANSN is 125 and 220 μM for AMC, it is expected that the ANSN substrate would have greater binding affinity for FXa (i.e., shorter lag times) than the AMC substrate. It has been suggested by Butenas et al. [9] that this is due to the limited possibility for modification of the AMC group. The lack of an adequate functional group does not allow for optimisation of substrate interaction compared to the presence of a modifiable sulfonyl moiety in the ANSN group which can enhance substrate specificity. Additionally, lag times were found to increase with increasing anticoagulant concentration. This shows the ability of FXa inhibitors to form a complex with ATIII and FXa. Any FXa not neutralised is free to react with the fluorogenic substrates; therefore lag times can be due to the time taken for the concentrations of the intermediate enzyme–substrate and enzyme–product complexes to rise to their steady-state levels [25].

And finally, the rates of the enzymatic reaction (i.e., rate of fluorophore formation) were higher for the AMC-based fluorogenic anti-FXa assay in all cases. In the presence of enoxaparin the AMC-based fluorogenic assay resulted in reaction rates that were between 3.6- and 4.8-fold higher than the ANSN assay except for 0.2 U mL^{-1} which was 1.8-fold higher. Tinzaparin reaction rates were between 2.3- and 8.4-fold higher than ANSN rates, and finally, danaparoid resulted in rates of reaction that were 2.6- to 5.1-fold higher than those in the ANSN assay. This behaviour is related to the catalytic constant (k_{cat}) values of FXa with respect to the two synthetic peptide substrates. The k_{cat} value for the ANSN substrate is 36 and 162 s^{-1} for the AMC substrate. Thus, the higher the k_{cat} value, the faster the enzyme–substrate complex conversion to fluorophore. Hence, these values indicate that FXa should convert the AMC-based fluorogenic substrate into its fluorophore 4.5-fold quicker than the ANSN-based fluorogenic substrate. This explanation is consistent with our findings.

In terms of assay sensitivity and reproducibility, the AMC-based substrate offers a wider assay sensitive range for enoxaparin, tinzaparin, and danaparoid compared with the ANSN detecting group in the fluorogenic anti-FXa assay. The ANSN-based (AMC-based) assay resulted in detection ranges of 0–0.4 U mL^{-1} (0–0.8 U mL^{-1}) for enoxaparin, 0–0.4 U mL^{-1} (0–0.6 U mL^{-1}) for tinzaparin, and 0–0.2 U mL^{-1} (0–1 U mL^{-1}) for danaparoid. In addition, assay reproducibility for both LMWHs and danaparoid when using the ANSN-based substrate was <10.5%, while the AMC-based substrate returned CVs of <7%.

Assay sensitivity as well as assay precision can also be analysed in terms of the k_{cat}/K_m ("specificity constant") values of FXa with respect to the two fluorogenic substrates. FXa has a k_{cat}/K_m of 290,000 M^{-1} s^{-1} for the ANSN fluorogenic substrate and ~740,000 M^{-1} s^{-1} for the AMC substrate. The importance of k_{cat}/K_m is that it determines the specificity of an enzyme for competing substrates. Therefore taking into account these two values, FXa shows a 2.5-fold greater specificity for AMC than for ANSN which translates into a wider assay sensitive range and better assay reproducibility. Therefore, what appears to be a poor substrate in terms of affinity for FXa (i.e. Pefafluor FXa has a relatively high K_m of 220 μM

JrnlID 216_ArtID 4314_Proof# 1 - 14/10/2010

compared with 125 μM for SN-7), it actually shows an overall greater effectiveness for substrate hydrolysis. However, it has been suggested by Bromfield et al. [18] who performed competitive inhibition assays for FXa with the fluorogenic coumarin substrate Boc-IEGR-AMC, that substrates with low K_m values rather than high k_{cat}/K_m values may be better indicators of inhibitor potential for a peptidic sequence. Nevertheless, it is a complex area of research due to the open and flat architecture of the FXa active site [26, 27] which results in its low selectivity for peptide substrates [28].

Overall, fluorogenic substrates incorporating the AMC leaving group are commonly used probes in proteolytic assays [29], fluorogenic thrombin generation assays [30, 31, 32, 33, 34] and more specifically in fluorogenic FXa assays [35, 36, 37, 38, 39]. The use of ANSN-based fluorogenic assays has also been described, but to a lesser extent for activated protein C [19], human factor VIIa and factor VIIa-tissue factor [40], and lately, for the *Mytilus edulis* anticoagulant peptide [41].

In summary, a new fluorogenic anti-FXa assay has been investigated in human pooled plasma based on the fluorescent reporter group ANSN. It was capable of statistically differentiating enoxaparin up to 0.4 U mL^{-1}, tinzaparin up to 0.4 U mL^{-1}, and danaparoid up to 0.2 U mL^{-1} with CVs of <10.5%. Moreover, it was compared with the 7-amino-4-methylcoumarin-based fluorogenic anti-FXa assay previously developed by Harris et al. [16]. Results indicate that the AMC-based fluorogenic anti-FXa assay is quicker, it has a wider sensitive range and it is more precise than the ANSN-based fluorogenic anti-FXa assay examined in this study.

Acknowledgment This work was supported by Enterprise Ireland under Grant No. TD/2009/0124.

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