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

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43	Abstract	<p>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 (C₂H₅)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-ϵ-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.</p>
44	Keywords separated by ' - '	FXa - Fluorogenic substrate - AMC - ANSN - Low molecular weight heparins - Danaparoid
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Comparative study of Factor Xa fluorogenic substrates and their influence on the quantification of LMWHs

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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 μ M 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 (Pefafleur 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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64 The endopeptidases involved in coagulation and fibrino- 117
 65 lysis are trypsin-like serine endopeptidases [9]. Research 118
 66 into the use of synthetic substrates for the evaluation of 119
 67 coagulation endopeptidases began in the 1950s and 1960s.
 68 Initially, chromogenic substrates based on the chromophore
 69 *p*-nitroaniline were developed for thrombin, plasmin, and
 70 trypsin analysis [8, 10], followed by the development of
 71 assays using fluorogenic substrates for thrombin [11, 12].
 72 Once the specific peptide cleavage sequence was known,
 73 these developments were rapidly extended to other coagu-
 74 lation proteins in the 70s and 80s, resulting in a new
 75 strategy for the biochemical study of the coagulation
 76 system [10].

77 Factor Xa occupies a critical position in the coagulation
 78 cascade; hence its joint popularity with thrombin as a
 79 therapeutic target for anticoagulant therapy. The first
 80 photometric anti-FXa assay was introduced in 1976 [13]
 81 followed by the rapid development of more specific
 82 chromogenic substrates and eventually a fluorogenic
 83 substrate specific for FXa which was introduced in 1977
 84 [14]. Both chromogenic and fluorogenic assays are well
 85 suited to the measurement of heparin, as there is a direct
 86 relationship between the functional activity of heparin and
 87 its effect on antithrombin allowing for greater precision in
 88 its determination [8]. Fluorescence-based measurements
 89 offer further advantages over colorimetric assays, such as
 90 superior sensitivity and specificity [14, 15], as well as
 91 measurement in a broader range of sample types such as
 92 platelet poor plasma, platelet rich plasma, and whole blood
 93 [16, 13].

94 Two fluorophores are principally used in commercial
 95 substrates for the analysis of coagulation proteins,
 96 namely 7-amino-4-methylcoumarin (AMC) and 6-amino-
 97 1-naphthalene-sulfonamide (ANSN) [17, 18, 19, 20]. The
 98 majority of studies have focussed on the reactivity of
 99 substrates containing 7-amino-4-methylcoumarin, as it is
 100 the most common fluorogenic leaving group used in
 101 bioassays [21]. Coumarins can be described as a broad class
 102 of fluorophores resulting in UV or near-UV excitation
 103 wavelengths with a high quantum yield and low extinction
 104 coefficient [22]. The naphthalenesulfonamides were reported
 105 in 1992 as a new fluorescent group for substrates of
 106 amidases which are also excited in the UV range. Their high
 107 quantum yield and the presence of a sulfonyl moiety allows
 108 for a range of chemical modifications which can enhance
 109 enzymatic substrate specificity [9]. However, given the
 110 availability of these substrates, few publications have
 111 evaluated their usefulness in the development of anti-FXa
 112 assays.

113 A novel fluorogenic anti-FXa assay was developed in
 114 this study using a peptide substrate for FXa with an
 115 attached ANSN fluorophore and was compared to a
 116 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

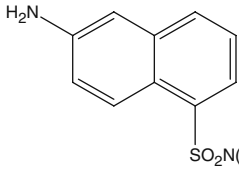
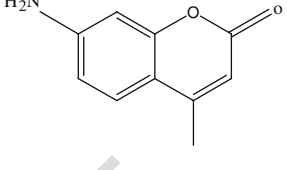
Reagents

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
 CaCl₂ from Fluka BioChemika (Buchs, Switzerland) was
 prepared from a 1 M CaCl₂ 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 (C₂H₅)₂ (SN-7), containing the fluores-
 cent 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), respec-
 tively. 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. (Männ-
 endorf, 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	Pefafneur FXa
Formula	Mes-D-LGR-ANSN(C ₂ H ₅) ₂	CH ₃ SO 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})

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

166 Fluorogenic anti-FXa assay

167 Measurements were carried out in reconstituted citrated
168 human pooled plasma. FXa and the ANSN-based fluoro-
169 genic substrate were titrated within the range of 0.1–
170 100 nM and 8.3–33.3 μM (K_m=125 μM), respectively.
171 Samples consisting of 6.25 μL of 100 mM CaCl₂, 43.75 μL
172 of pooled plasma, and 50 μL of FXa (0.1–100 nM) were
173 incubated at 37 °C for 3 min and shaken for the first 150 s.
174 The reaction was started by adding 50 μL of ANSN-based
175 fluorogenic substrate (8.3–33.3 μM). Samples within wells
176 were mixed with the aid of orbital shaking at 37 °C for
177 30 s. Finally, immediately after shaking, fluorescence
178 measurements were recorded at 37 °C for 60 min with
179 integration time of 20 μs. Fluorescence excitation was at
180 352 nm and emission was monitored at 470 nm,
181 corresponding to the excitation/emission wavelengths of
182 the ANSN fluorophore. All measurements were carried out
183 in triplicate. Following optimization of assay conditions,
184 pooled commercial plasma samples were spiked with
185 pharmacologically relevant concentrations (0–1.6 U mL⁻¹)
186 of therapeutic anticoagulants including enoxaparin, tinza-
187 parin, and danaparoid. The reaction rate (slope), which is
188 defined as the change in fluorescence divided by the change
189 in time (i.e., dF/dt), was measured as the linear portion of
190 the fluorescence response profile and plotted versus
191 anticoagulant concentration. The assay using the Pefafneur
192 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 com-
pared 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

218 of ANSN fluorophore release as a result of FXa substrate
 219 cleavage and is compared to the rate of AMC fluorophore
 220 release which has been published previously [16]. Opti-
 221 mised assay concentrations for this new fluorogenic assay
 222 were determined by performing titrations of FXa from 0.1
 223 to 100 nM and fluorogenic substrate concentrations from
 224 8.3 to 33.3 μM . To allow for differentiation in the assay
 225 reaction rates, the concentration range of the ANSN-based
 226 substrate was selected so that it was lower than the K_m
 227 value. The final working assay concentrations of 25 μM
 228 ANSN-based fluorogenic substrate and 100 nM FXa,
 229 conform to the optimisation criteria which included short
 230 lag times, fast reaction rates, and a broad fluorescence
 231 dynamic range for the differentiation of anticoagulant
 232 concentrations. The same criteria were also selected for
 233 the optimization of the AMC-based fluorogenic anti-FXa
 234 assay.

235 Evaluation of the ANSN-based anti-FXa fluorogenic assay

236 Three anticoagulant drugs were tested using the fluorogenic
 237 anti-FXa assay with the ANSN-based substrate in commer-
 238 cial pooled plasma.

239 The fluorescence intensity profiles over time for tinza-
 240 parin plasma samples in the fluorogenic anti-FXa assay can
 241 be seen in Fig. 1 and the inset shows the dose–response
 242 profile versus anticoagulant concentration. As the concen-
 243 tration of tinzaparin increased, the fluorescence profiles
 244 reached lower fluorescence intensity values after 1 h. In the
 245 absence of tinzaparin, the maximum intensity value was
 246 56,000 AU; with 0.2 U mL^{-1} the profile reached
 247 40,000 AU and 27,000 AU for 0.4 U mL^{-1} . From 0.4 to
 248 1.6 U mL^{-1} , the maximum fluorescence intensity value was
 249 between 22,000 and 27,000 AU.

250 Lag times were calculated by extrapolation of the
 251 linear portion of the progression curve to its intersection
 252 with the x-axis [23]. In the presence of higher anticoag-
 253 ulant concentrations, longer lag time values were ob-
 254 served. At low concentrations of 0 and 0.2 U mL^{-1}
 255 tinzaparin, the reaction was very quick and no lag
 256 time was observed. Lag times increased to 310 and
 257 630 s for 0.4 U mL^{-1} and 0.6 U mL^{-1} respectively. At
 258 concentrations of 0.8–1.6 U mL^{-1} lag time values were
 259 1,010 s.

260 The reaction rates of the fluorescence profiles were
 261 calculated to generate dose–response profiles and a similar
 262 trend was observed for all drugs analysed, in that as
 263 anticoagulant concentration increased, the rate of the
 264 reaction decreased. The slope values were statistically
 265 different up to 0.4 U mL^{-1} ($p < 0.003$) with significantly
 266 reduced sensitivity in the upper tinzaparin range (0.6–
 267 1.6 U mL^{-1}).

268 The anti-FXa assay was also tested with enoxaparin,
 269 another member of the low molecular weight heparin
 270 family. The fluorescence profiles are very similar to those
 271 of tinzaparin (Fig. 2). The fluorescence profiles for
 272 0 U mL^{-1} reached 52,000 AU after 1 h, for 0.2 U mL^{-1}
 273 the intensity value was 38,000 AU and with 0.4 U mL^{-1} the
 274 profile reached 28,000 AU. At higher enoxaparin concen-
 275 trations, the profiles reached maximum intensity values of
 276 21,000 and 28,000 AU.

277 At low concentrations of enoxaparin lag times were
 278 absent but were calculated as 110 s for 0.4 and 0.6 U mL^{-1}
 279 enoxaparin. At higher concentrations, lag times increased to
 280 590 s for 0.8 and 1 U mL^{-1} , 730 s for 1.2 U mL^{-1} , and
 281 910 s for 1.4 and 1.6 U mL^{-1} .

282 The dose–response curve calculated from the linear
 283 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

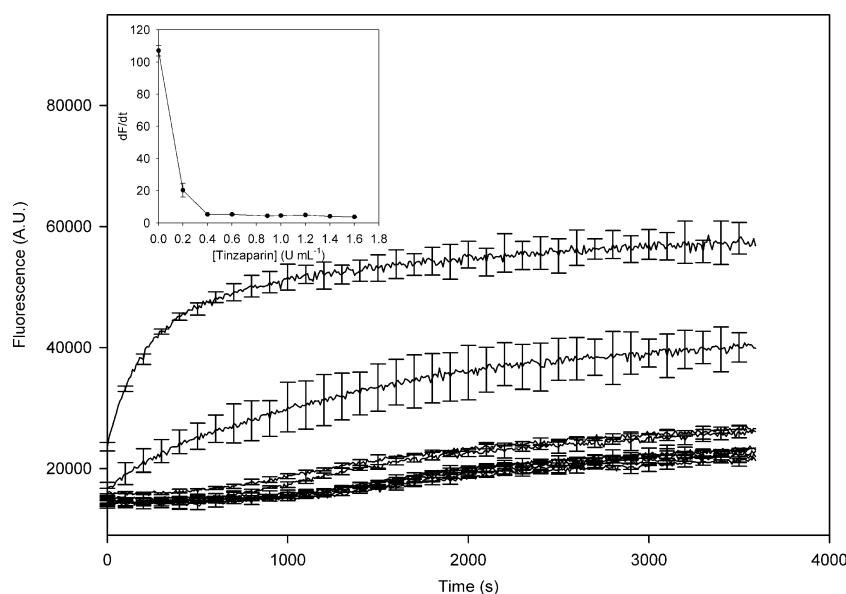
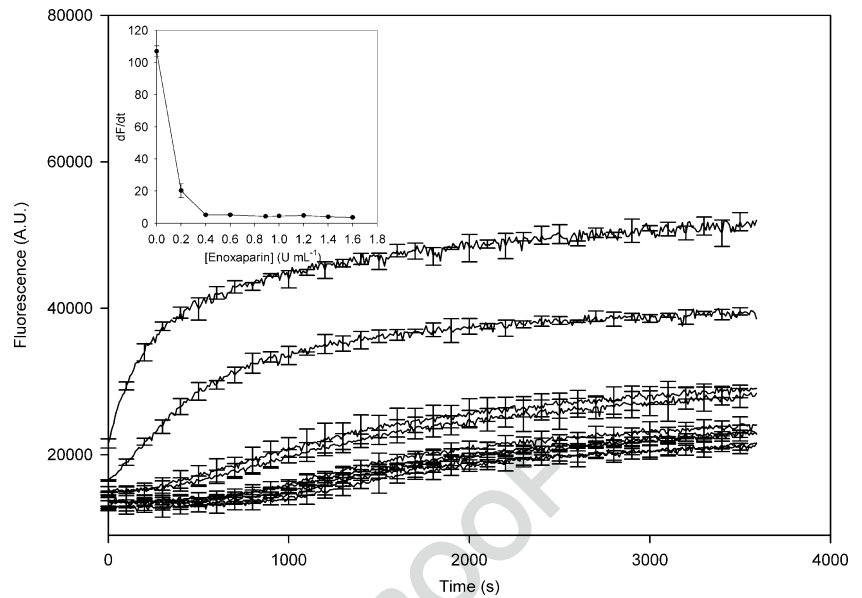


Fig. 2 Fluorescence intensity vs. time for the anti-FXa activity with enoxaparin ($n=3$). Enoxaparin concentration increases from top to bottom. *Inset* Dose-response calibration curve of enoxaparin in pooled plasma in the ANSN-based fluorogenic anti-FXa assay ($n=3$), calculated from the linear slopes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



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

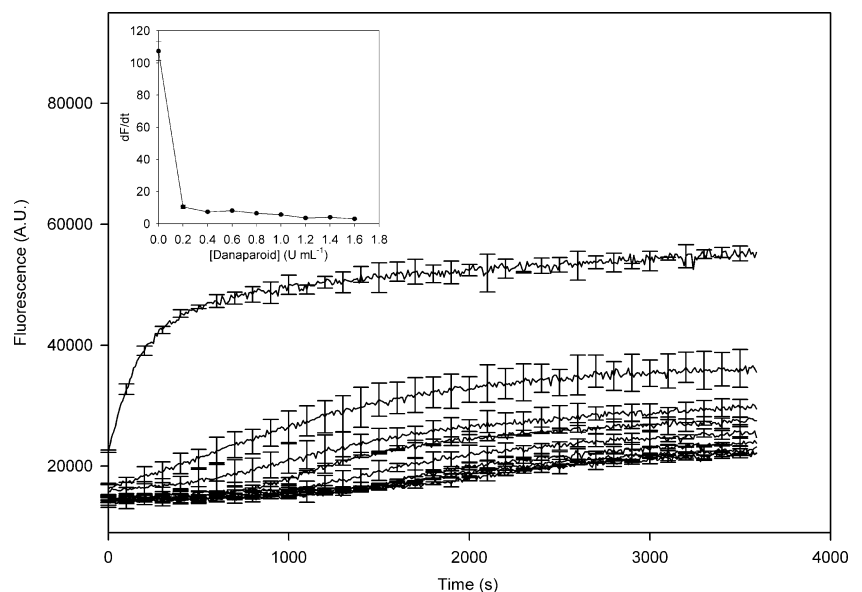
286 Figure 3 shows the fluorescence profiles of the anti-Xa
287 assay in the presence of danaparoid. The fluorescence
288 profile for 0 U mL^{-1} reached 55,000 AU after 1 h, at
289 0.2 U mL^{-1} the maximum fluorescence intensity value was
290 35,000 AU and from 0.4 to 1.6 U mL^{-1} danaparoid,
291 profiles reached fluorescence intensities between 22,000
292 and 29,000 AU.

293 Similar to the LMWHs, lag times were not observed at
294 low concentrations of danaparoid. Lag times increased
295 from 200 s at 0.4 U mL^{-1} to 780 s at 0.6 U mL^{-1} , 1,050 s
296 at 0.8 U mL^{-1} and 1180 s at 1 U mL^{-1} . At 1.2 U mL^{-1} and
297 1.6 U mL^{-1} danaparoid lag times were 1,320 s and at
298 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–
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



314 all drugs tested and only at concentrations of 0.6, 0.8, and
 315 1 U mL⁻¹, were tinzaparin reaction rates lower than
 316 danaparoid, but these fall into the statistically insensitive
 317 range.

318 The analytical errors (standard deviation and percentage
 319 coefficient of variation) related to each of the slope
 320 measurements for all three anticoagulants were calculated.
 321 All CV values were <10.5% with the exception of
 322 0.2 U mL⁻¹ tinzaparin which returned a CV value of 21%.

323 Comparative studies of AMC versus ANSN-based
 324 fluorogenic assays

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

329 *Fluorescence intensity*

330 All three of the AMC-based fluorogenic assay reaction
 331 progress curves for enoxaparin, tinzaparin, and danaparoid,
 332 reported by Harris et al. [16], approached a similar
 333 maximum plateau value of product formation at approx-
 334 imately the same level (i.e., 48,000–53,000 AU) indepen-
 335 dent of anticoagulant type and concentration. This is an
 336 indication of substrate depletion and also that the enzyme
 337 remains stable under the conditions tested. In the ANSN-
 338 based study, the maximum value of product formed for all
 339 three anticoagulant drugs reached different fluorescence
 340 intensity values depending on anticoagulant concentration.
 341 As the concentration of anticoagulant increased from 0 to
 342 1.6 U mL⁻¹, the fluorescence intensity values decreased
 343 from 56,000 to 22,000 for tinzaparin, 52,000 to 21,000 for
 344 enoxaparin and finally, from 55,000 to 22,000 for
 345 danaparoid.

346 *Lag time values*

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

353 *Slope values*

354 Analysis of slope values at 0 U mL⁻¹ returned 25%
 355 variation in all assays. As a result, all slope values were
 356 normalised with respect to those at 0 U mL⁻¹ for each
 357 anticoagulant. As can be seen in Fig. 4, the fluorogenic
 358 anti-FXa assay using the AMC fluorogenic substrate

returned higher slopes than the ANSN fluorogenic assay 359
 in all instances. The inter-assay variability between the two 360
 fluorogenic substrates for each anticoagulant drug was 361
 statistically evaluated using the paired Student's *t* test. 362
 Results indicate that there is a significant mean difference 363
 (*p*<0.05) between the two fluorogenic anti-FXa assays for 364
 each anticoagulant at all concentrations, except for 365
 0 U mL⁻¹ as it corresponds to the unit value of normal- 366
 isation in all cases. 367

Assay sensitivity 368

The intra-assay variability between different concentrations 369
 of anticoagulants was assessed for both fluorogenic 370
 substrates. Table 3 outlines the statistically sensitive range 371
 for both fluorogenic anti-FXa assays each containing a 372
 different leaving group in the presence of tinzaparin, 373
 enoxaparin, and danaparoid. When the ANSN-based fluo- 374
 rogenic anti-FXa assay was performed in the presence of 375
 tinzaparin, the slopes of the enzymatic reaction were 376
 statistically different up to 0.4 U mL⁻¹ (*p*<0.05) at intervals 377
 of 0.2 U mL⁻¹. In the case of the Pefafloor FXa substrate 378
 the assay proved to be sensitive up to 0.6 U mL⁻¹ 379
 tinzaparin (*p*<0.001). This reduction in assay sensitivity 380
 for the ANSN-based fluorogenic anti-FXa assay was also 381
 observed for enoxaparin. The fluorogenic anti-FXa assay 382
 using the ANSN-based substrate resulted in a narrower 383
 statistically sensitive range from 0 to 0.4 U mL⁻¹ (*p*<0.05) 384
 when compared with the AMC-based substrate assay range 385
 of 0–0.8 U mL⁻¹ (*p*<0.001) for enoxaparin. The effect of 386
 danaparoid was also evaluated in both fluorogenic assays 387
 and a larger difference in assay sensitivity was observed. 388
 Response slopes to danaparoid concentrations at 389
 0.2 U mL⁻¹ intervals were statistically different up to 390
 0.2 U mL⁻¹ and 1 U mL⁻¹ for the ANSN and AMC-based 391
 substrate assays, respectively. In all cases, the AMC-based 392
 substrate seems to offer a wider assay sensitive range 393
 compared with the ANSN-based fluorogenic substrate 394
 when quantifying both LMWHs and danaparoid. 395

Reproducibility (%CV values) 396

Reproducibility of the two developed assays was deter- 397
 mined using the percentage coefficient of variation (%CV). 398
 Comparing CV values between the two fluorogenic assays, 399
 results in lower variation for both low molecular weight 400
 heparins and danaparoid with the AMC fluorophore. 401
 Studies of precision for the fluorogenic anti-FXa assay 402
 using the AMC-based substrate showed a coefficient of 403
 variation ranging between 0.5% and 7% [16]. In the case of 404
 the ANSN-based assay, CV values were below 10.5% 405
 except for 0.2 U mL⁻¹ tinzaparin which returned a CV of 406
 21%. 407

Comparative study of Factor Xa fluorogenic substrates

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	t2.2
0.2	280	0	240	0	560	0	t2.3
0.4	420	110	380	310	800	200	t2.4
0.6	580	110	670	630	1,420	780	t2.5
0.8	850	590	900	1,010	2,010	1,050	t2.6
1	940	590	1,040	1,010	2,070	1,180	t2.7
1.2	1,020	730	1,440	1,010	2,440	1,320	t2.8
1.4	1,260	910	1,800	1,010	>3,600	1,480	t2.9
1.6	1,310	910	2,000	1,010	>3,600	1,320	t2.10

^a Averaged of triplicate measurements (n=3)

408 **Discussion**

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

424 A number of studies have been devoted to the
 425 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 fluoro-
 genic 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 fluo-
 rogenic 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)

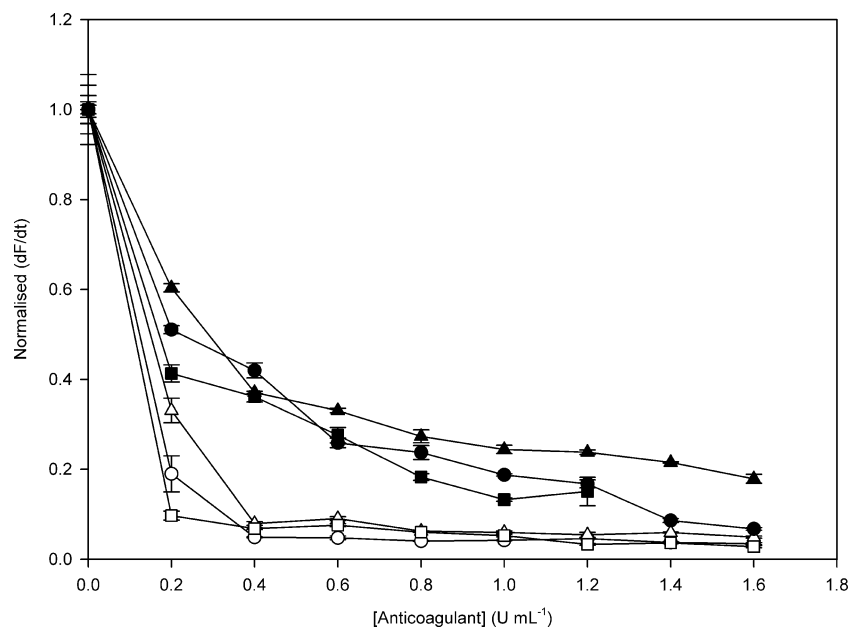


Table 3 Comparison of the statistically sensitive range for each anticoagulant tested in two fluorogenic anti-FXa assays based on different fluorophore leaving groups

Anticoagulant drug	Statistically sensitive range (U mL ⁻¹)	
	ANSN	AMC
Tinzaparin	0–0.4	0–0.6
Enoxaparin	0–0.4	0–0.8
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. Peflafluor FXa has a relatively high K_m of 220 μM

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

551 Overall, fluorogenic substrates incorporating the AMC
 552 leaving group are commonly used probes in proteolytic
 553 assays [29], fluorogenic thrombin generation assays [30,
 554 31, 32, 33, 34] and more specifically in fluorogenic FXa
 555 assays [35, 36, 37, 38, 39]. The use of ANSN-based
 556 fluorogenic assays has also been described, but to a lesser
 557 extent for activated protein C [19], human factor VIIa and
 558 factor VIIa-tissue factor [40], and lately, for the *Mytilus*
 559 *edulis* anticoagulant peptide [41].

560 In summary, a new fluorogenic anti-FXa assay has been
 561 investigated in human pooled plasma based on the
 562 fluorescent reporter group ANSN. It was capable of
 563 statistically differentiating enoxaparin up to 0.4 U mL⁻¹,
 564 tinzaparin up to 0.4 U mL⁻¹, and danaparoid up to
 565 0.2 U mL⁻¹ with CVs of <10.5%. Moreover, it was
 566 compared with the 7-amino-4-methylcoumarin-based fluo-
 567 rogenic anti-FXa assay previously developed by Harris et
 568 al. [16]. Results indicate that the AMC-based fluorogenic
 569 anti-FXa assay is quicker, it has a wider sensitive range and
 570 it is more precise than the ANSN-based fluorogenic anti-
 571 FXa assay examined in this study.

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