**Comparison of the anticoagulant response of a novel fluorogenic anti-FXa assay with two commercial anti-FXa chromogenic assays**

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**Abstract**

*Introduction:* Fast and accurate monitoring is crucial in the successful regulation of coagulation therapy. For the treatment of venous thromboembolism, both unfractionated heparin (UFH) and low molecular weight heparins (LMWH) are commonly administered. The chromogenic anti-factor Xa (FXa) assay is currently considered the 'gold standard' assay for monitoring LMWH. However different commercial chromogenic methods often differ when tested with the same samples. Fluorogenic anti-FXa assays have the potential to offer greater benefits over chromogenic assays in terms of greater specificity, sensitivity and they are not so influenced by sample opacity or turbidity.

*Materials and Methods:* Commercial plasmas were spiked with pharmacologically relevant concentrations (0–1 U/ml) of UFH, enoxaparin, and tinzaparin. The fluorogenic assay was carried out using previously optimized concentrations of 4 nM FXa and 0.9 µM fluorogenic substrate, in addition to 6.25 µl of 100 mM $CaCl_2$ and 43.75 µl of plasma. The Biophen® and Coamatic chromogenic assays were carried out according to the manufacturer's instructions. Reaction rates and endpoint values were analyzed and statistical analysis by means of one-way analysis of variance (ANOVA) was performed.

*Results:* The fluorogenic anti-FXa assay was found to have the broadest therapeutic range of 0-1 U/ml with CVs of < 5% for UFH and tinzaparin and CVs < 9% for enoxaparin. Despite their limited measuring range, good assay reproducibility was observed with both chromogenic kits.

*Conclusions:* This study indicated that the fluorogenic assay is the most sensitive assay with the broadest dynamic range for monitoring LMWH therapy when compared with standard chromogenic assays.

*Keywords:* Factor Xa; Fluorogenic; Chromogenic; Low molecular weight heparin

*Abbreviations:*

7-amino-4-methylcoumarin (AMC)

Activated clotting time (ACT)

Activated partial thromboplastin time (APTT)

Antithrombin (AT)

Arbitrary units (AU)

Coefficient of variation (CV)

Factor Xa (FXa)

Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

Low molecular weight heparin (LMWH)

One way analysis of variance (ANOVA)

Platelet poor plasma (PPP)

Platelet rich plasma (PRP)

*p*-nitroaniline (pNA*)*

Unfractionated heparin (UFH)

## Introduction

Anticoagulants including unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) are commonly administered to patients for the treatment of cardiovascular diseases such as arterial thromboembolism and coronary artery disease [1-3]. While UFH can be monitored using conventional clot-based says such as the activated partial thromboplastin time (APTT) and activated clotting time (ACT), these tests cannot be used to accurately determine LMWH activity [3-6]. However, the development of anti-factor Xa (FXa) assays and their use in central diagnostic laboratories has allowed for more accurate and sensitive monitoring of LMWH therapy [5, 7, 8].

The standard anti-FXa assays currently used for clinical monitoring of LMWH are chromogenic-based assays [9, 10]. The introduction of synthetic substrates for the testing of serine proteases and their inhibitors began in the 1950s [11]. In 1972 oligopeptide *p*-nitroanilides were developed, which were proven to be sensitive to thrombin, plasmin, and trypsin [12]. These oligopeptide substrates were coupled to the chromophore *p*-nitroaniline (pNA) via an amide linkage so that the protease to be assayed could hydrolyze the chromogenic tripeptide-pNA, releasing the yellow pNA for photometric detection at 405 nm [11]. Research into synthetic substrates continued and the first anti-FXa chromogenic assay was developed by Teien in 1976. It utilised FXa and the chromogenic substrate Bz-Ile-Glu-Gly-Arg-pNA in a simple two-stage assay, allowing for the determination of FXa activity through substrate amidolysis. The accuracy and precision of this newly developed assay was comparable to that of existing clotting assays in use, resulting in its adaptation as the standard assay for monitoring LMWH [13-15].

Although chromogenic assays confer many advantages over standard clot-based assays, such as their increased sensitivity to LMWHs, they do have several limitations including poor comparability between commercially available anti-FXa chromogenic assays, differences in ratios of anti-FXa to anti-FIIa among the various LMWH preparations, and the variability caused by the timing of blood sampling in relation to dosing [16, 17]. As the testing method relies on optical density readings, it requires samples to be relatively clear which precludes the use of whole blood and platelet rich plasma (PRP) samples [18]. This problem is also encountered in the presence of fibrinogen clotting, as the increased turbidity of the sample interferes negatively with the absorbance readings [13, 18, 19].

With fluorogenic assays on the other hand, it is possible to test a range of sample types such as platelet poor plasma (PPP), PRP, and whole blood samples, as fluorescence is not influenced by sample opacity [19, 20]. Fluorogenic assays became increasingly popular for proteolytic assays in the 1970s [21] and several fluorogenic substrates for both thrombin and FXa were developed [22, 23]. However the development of chromogenic assays prior to the advent of fluorogenic substrates resulted in the wide availability of colorimeters in diagnostic laboratories. The ease of availability of these assays, cost, and instrumentation availability favoured the use of chromogenic substrates which is why routine fluorogenic methods were not readily adapted [24].

In this study we assessed if the novel fluorogenic anti-FXa assay previously developed in our laboratory [20] would compare with two commercially available anti-FXa chromogenic assays, when tested with pooled human plasma containing therapeutic concentrations of UFH and two LMWHs.

## 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 stock 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 aluminum foil to protect from exposure to light. Purified human FXa (serine endopeptidase; code number: EC 3.4.21.6) was obtained from HYPHEN BioMed (Neuville-Sur-Oise, France) and was reconstituted in 1 ml of PCR grade water to give a stock concentration of 2200 nM.

The Biophen® Heparin Anti-Xa chromogenic kit was purchased from Hyphen BioMed (Neuville-Sur-Oise, France) and the Coamatic® Heparin chromogenic kit was obtained from Chromogenix (Milano, Italy). Unfractionated heparin (sodium salt of heparin derived from bovine intestinal mucosa, H0777) was sourced from Sigma-Aldrich (Dublin, Ireland), Tinzaparin (Innohep®) and Enoxaparin (Clexane®) were obtained from LEO Pharma (Ballerup, Denmark) and Sanofi-Aventis (Paris, France) respectively. 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.

*Apparatus and software*

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

*Fluorogenic anti-FXa assay*

All measurements for the fluorogenic anti-FXa assay were carried out in reconstituted citrated human pooled plasma without the addition of exogenous AT. Pooled commercial plasma samples were spiked with pharmacologically relevant concentrations (0–1 U/ml) of therapeutic anticoagulants including UFH, enoxaparin, and tinzaparin. FXa and Pefafluor FXa fluorogenic substrate concentrations were previously optimized as 4 nM and 0.9 µM respectively for the fluorogenic anti-FXa assay [20]. Each well contained 6.25 µl of 100 mM $CaCl_2$, 43.75 µl of pooled plasma, and 50 µl of FXa. The reaction was started by adding 50 µl of Pefafluor FXa fluorogenic substrate. Samples within wells were mixed with the aid of orbital shaking at 37 °C for 30 s. Immediately after shaking, fluorescence measurements were recorded at 37 °C for 60 min, with a 20 µs integration time. Fluorescence excitation was at 342 nm and emission was monitored at 440 nm, corresponding to the excitation/emission wavelengths of the 7-amino-4-methylcoumarin (AMC) fluorophore. All the measurements were carried out in triplicate.

*Commercial chromogenic assays*

All measurements for the chromogenic anti-FXa assays were carried out in reconstituted citrated human pooled plasma. Pooled commercial plasma samples were spiked with pharmacologically relevant concentrations (0–1 U/ml) of therapeutic anticoagulants including UFH, enoxaparin, and tinzaparin. The Biophen® Heparin chromogenic assay was carried out according to the manufacturer's instructions as follows: each well contained 50 µl of plasma and 50 µl of antithrombin (AT). To this, 50 µl of FXa was added. The reaction was started by adding 50 µl of FXa specific chromogenic substrate. Samples within wells were mixed within the spectrophotometer by orbital shaking at 37 ºC for 30 s. Immediately after shaking, absorbance measurements were recorded at 37 ºC for 60 min, at 10 s intervals. Absorbance was measured at 405 nm and all measurements were performed in triplicate. The exact same procedure was followed for the Coamatic® Heparin chromogenic assay without the addition of 50 µl of AT.

*Data and statistical analysis*

All graphs were plotted using SigmaPlot 8.0. Data generated from the fluorogenic and chromogenic anti-FXa assays were plotted as absorbance/fluorescence intensity versus time. The analytical parameter for the fluorogenic assay was defined as the reaction rate (slope), which can be described as the change in fluorescence divided by the change in time (i.e. dF/dt). This is the linear portion of the fluorescence response profile and is plotted against different anticoagulant concentrations to generate a dose-response curve. Following analysis of the response profiles for both the commercial chromogenic assays, it was clear that the reaction rate was an unsuitable analytical parameter, and therefore the endpoint value was selected to construct dose-response curves for these assays.

SPSS 17.0 was used for statistical analysis and all data was transformed logarithmically prior to analysis. Intra-assay differences between anticoagulant concentrations were compared using one-way analysis of variance (ANOVA), with subsequent post-hoc analysis (Scheffe test, Tukey's test, and Duncan's test) if significance was observed. A result of $p<0.05$ was considered statistically significant. Assay comparisons were then performed based on the sensitivity and responsiveness of each assay as established by the intra-assay statistical analysis.

**Results**

All three anticoagulants tested in the fluorogenic anti-FXa assay resulted in similar fluorescence profiles so a representative graph is shown in Fig. 1. As can be seen in Fig. 1, all profiles at each concentration reached maximum plateau between 20000 and 25000 arbitrary units (AU), the lag time increased with increasing anticoagulant concentration and the slope of the curve decreased with increasing concentration.

Analysis of the normalized dose-response curves in Fig. 2, which were generated from the initial reaction rates of the profiles, showed that the fluorogenic anti-FXa assay was sensitive to UFH, tinzaparin, and enoxaparin from 0 U/ml to 1 U/ml. Statistical analysis of the logarithmically transformed data using one-way ANOVA, proved that the assay was capable of differentiating all anticoagulant concentrations at intervals of 0.2 U/ml from 0 to 1 U/ml ($p<0.05$), indicating a high degree of assay sensitivity across the dynamic range. The assay was reproducible with CVs of < 5% for UFH and tinzaparin and < 9% CV for enoxaparin.

The absorbance profiles generated by the chromogenic assays differed from the profiles generated by the fluorogenic assay. While the fluorescence profiles at each concentration began with a short lag time, rapid increase and plateau at the same level, the chromogenic profiles lacked a lag time, they demonstrated a decreasing reaction rate with increasing anticoagulant concentration and as concentration increased each progress curve reached a different level of absorbance intensity. An example of this can be seen in Fig. 3.

Visual analysis of the absorbance profiles at increasing concentrations of UFH in the Biophen® chromogenic assay (Fig. 3) indicated a high degree of sensitivity to UFH over the dynamic range. In Fig. 4 the dose-response curve based on the extracted endpoint values can be seen.

Logarithmic transformation of the endpoint values returned statistically significant differences between concentrations from 0 to 1 U/ml for UFH ($p<0.05$). Analysis of the tinzaparin dose-response profile in Fig. 4 indicates high assay sensitivity at low concentrations of tinzaparin but at high concentrations the assay becomes less sensitive. Statistical analysis, using one-way ANOVA followed by post-hoc analysis, of the endpoint values returned significant differences ($p<0.05$) between concentrations up to 0.6 U/ml. Statistical analysis of the dose-response profile for enoxaparin in the Biophen® chromogenic assay (Fig. 4) indicated that the differences recorded between plasma samples up to 0.4 U/ml were significant ($p<0.05$). Assay reproducibility was very good with CVs of < 5% for all drugs tested.

The absorbance profiles generated by plasma samples spiked with UFH from 0 to 1 U/ml using the Coamatic® chromogenic assay are shown in Fig. 5. Using the endpoint values, a dose-response curve (Fig. 6) was generated. Logarithmic transformation of the endpoint values resulted in statistically significant differences ($p<0.05$) between 0 and 0.4 U/ml for UFH. Statistical analysis of the dose-response curve (Fig. 6) indicated sensitivity to tinzaparin and enoxaparin up to 0.6 U/ml and 0.4 U/ml respectively. Assay reproducibility was < 5% were for all drugs tested.

All assays returned CVs of < 9%, but the fluorogenic anti-FXa assay returned the broadest dynamic range. A summary of the statistically sensitive ranges for all drugs tested with each assay and the reproducibility of each is outlined in Table 1.

## Discussion

Chromogenic anti-FXa assays are currently the 'gold standard' for monitoring UFH and LMWH therapy in patients suffering from thrombotic disorders [16, 25, 26]. Chromogenic assays were first introduced in the 1960s with the development of synthetic peptide substrates for various coagulation proteases such as thrombin and FXa [11-13]. Greater reproducibility was achieved with these assays than had previously been observed with traditional clot-based assays, hence their immediate uptake into central haemostasis laboratories [14]. It has been further suggested that the way forward is to monitor anti-FXa levels in rapid assay formats developed specifically for point-of-care testing [5].

In this study the aim was to compare a novel fluorogenic anti-FXa assay to currently available commercial anti-FXa chromogenic kits in a bid to ascertain if the fluorogenic anti-FXa assay could offer a similar or greater level of sensitivity to the anticoagulants under evaluation. The fluorogenic anti-FXa assay was compared with two commercially available chromogenic assay kits; the Biophen® and the Coamatic® chromogenic kits. All assays were performed using human pooled plasma samples spiked with therapeutic concentrations of UFH and two LMWHs, enoxaparin, and tinzaparin. The intra-assay variability and dynamic ranges for these three drugs was established for each assay. Different analytical parameters were selected to determine the intra-assay variability for the chromogenic and fluorogenic assays. The responses of the fluorogenic and chromogenic assays to the three different drugs were then compared in terms of dynamic range.

From the results obtained in this study, it was established that the FXa fluorogenic assay had the broadest dynamic range for each drug. UFH was detected up to a concentration of 1 U/ml in both the fluorogenic anti-FXa assay and the Biophen®

chromogenic assay, with much lower levels of sensitivity up to 0.4 U/ml with the Coamatic® kit. Tinzaparin was detected up to a concentration of 1 U/ml in the fluorogenic assay and to a concentration of 0.6 U/ml in both commercially available chromogenic assay kits. The smallest dynamic range for all assays, with the exception of the fluorogenic anti-FXa assay, was for enoxaparin. While the drug could be detected using the fluorogenic assay to 1 U/ml, both commercially available chromogenic assay kits could only detect up to levels of 0.4 U/ml. According to the manufacturer's datasheets, both the Coamatic® and Biophen® kits are purported to be sensitive to anticoagulant (UFH and LMWH) concentrations of 1.5 U/ml and 1 U/ml respectively with CVs of <6%. However, when performed as part of this study, while these assays were quite reproducible with CVs of <5%, they failed to reach their suggested therapeutic ranges, rendering the fluorogenic assay superior to the chromogenic assay in terms of its response to the anticoagulants tested.

While significant variability in sensitivity between different coagulation methods (clot-based, chromogenic, and point-of-care) have been reported [3, 27, 28] significant differences often arise even when the method of testing is identical but the manufacturer of the test kit differs.

For example, many studies have compared the responses of different anti-FXa chromogenic-based assays [16, 17, 29, 30]. In one such study, anti-FXa levels were compared using three different commercial chromogenic kits [17]. Results showed that even when performed according to the specifications of the manufacturer, different chromogenic methods returned significantly different anti-FXa levels for the same patient sample [17]. Kitchen et al. also investigated and compared five different chromogenic assays and observed a difference of > 0.25 U/ml in mean UFH levels between assays [30].

In the measurement of anti-FXa levels, variability in results for different chromogenic assays is common. In a comment to the editor of Chest in 2002, Smythe et al. [31] reported a mean difference of as high as 0.16 U/ml in heparin levels as deduced by anti-FXa analysis. Such differences are attributable to instrument and assay variability. It has thus been suggested that the therapeutic heparin range, as determined by anti-FXa assays, be instrument and assay specific [17, 31, 32]. Another study was also carried out comparing two chromogenic anti-FXa assays [16], the COATEST® and MODIFIED COAMATIC® assay from Chromogenix, and significant differences were observed between both methods in the high-dose UFH setting. The results again indicated that the choice of method used in a clinical setting requires careful consideration. It has been suggested that differences in the measurement of anti-FXa levels may be due to varying sensitivities of each specific method to the AT present endogenously in the sample. Therefore the addition of excess AT may aid in sample reproducibility [17].

Turbidity is a contributing factor to this variability in coagulation testing, especially in relation to chromogenic testing. Lyophilization of commercial plasmas induces turbidity due to the presence of lipid containing complexes, chylomicrons and very low density lipoproteins [33]. Fluorogenic assays however, are not influenced by sample opacity and therefore a range of sample types such as PRP and whole blood with minimal sample preparation required can be used with this assay [7, 20]. However it must be noted that there are limitations with fluorogenic substrates where the rate of product formation is not necessarily proportional to the enzyme concentration, but this can be overcome by using substrates that are not significantly consumed. Also fluorescence intensity may not be proportional to the concentration of fluorophore due to the inner filter effect or absorption of light at both excitation and

emission wavelengths. This can be addressed by sample dilution or correcting mathematically referring to the measured absorbance of the sample [18].

Marked differences in the response of the two chromogenic assays to UFH and LMWH were observed in the study presented here, which can be corroborated by previous studies [17, 30]. The fluorogenic anti-FXa assay displayed a greater dynamic and sensitive range compared to the commercial chromogenic assays when tested with pooled plasma samples containing different anticoagulants.

To summarise, it has been established that the fluorogenic anti-FXa assay exhibits a broad dynamic range with both UFH and LMWHs. This sensitive range coupled with the potential of the assay to test more complex sample types than clot-based or chromogenic assays [7, 20], indicates that with further development, fluorogenic anti-FXa assays could become a successful method for anticoagulant monitoring.

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**Tables**

| Assay | Anticoagulant drug | Sensitive range (U/ml) | % CV |
|---|---|---|---|
| Fluorogenic | UFH | 0-1 | < 5% |
| | Tinzaparin | 0-1 | < 5% |
| | Enoxaparin | 0-1 | < 9% |
| Chromogenic | UFH | 0-1 | < 5% |
| Biophen® | Tinzaparin | 0-0.6 | < 5% |
| | Enoxaparin | 0-0.4 | < 5% |
| Chromogenic | UFH | 0-0.4 | < 5% |
| Coamatic® | Tinzaparin | 0-0.6 | < 5% |
| | Enoxaparin | 0-0.4 | < 5% |

## Table Legends

Table 1. Comparison of the statistically sensitive range for each anticoagulant tested in the three anti-FXa assays evaluated and the variability associated with each.

**Figure Legends**

Fig. 1: Fluorescence profiles of the fluorogenic anti-FXa assay in the presence of UFH (0-1 U/ml) (arrow indicates increasing UFH concentration).

Fig. 2: Normalised dose-response curves of the fluorogenic anti-FXa assay in the presence of UFH (●), tinzaparin (▲) and enoxaparin (■).

Fig. 3: Absorbance profiles of the Biophen® chromogenic anti-FXa assay in the presence of UFH (0-1 U/ml) (arrow indicates increasing UFH concentration).

Fig. 4: Normalised dose-response curves of the Biophen® chromogenic anti-FXa assay in the presence of UFH (●), tinzaparin (▲) and enoxaparin (■).

Fig. 5: Absorbance profiles of the Coamatic® chromogenic anti-FXa assay in the presence of UFH (0-1 U/ml) (arrow indicates increasing UFH concentration).

Fig. 6: Normalised dose-response curves of the Coamatic® chromogenic anti-FXa assay in the presence of UFH (●), tinzaparin (▲) and enoxaparin (■).