

Comparison of a fluorogenic anti-FXa assay with a central laboratory chromogenic anti-FXa assay for measuring LMWH activity in patient plasmas

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Abstract

Introduction: Low molecular weight heparins (LMWHs) are used worldwide for the treatment and prophylaxis of thromboembolic disorders. Routine laboratory tests are not required due to the predictable pharmacokinetics of LMWHs, with the exception of pregnant patients, children, patients with renal failure, morbid obesity, or advanced age. Anti-Factor Xa (anti-FXa) plasma levels are most often employed in the assessment and guidance of accurate dosing in these patient cohorts.

Materials and methods: A LMWH calibration curve was generated using citrated human pooled plasma spiked with pharmacologically relevant concentrations (0–1.2 U/ml) of two low molecular weight heparins; enoxaparin and tinzaparin. Least squares analysis determined the best curve fit for this set of data which returned low sum of squares (SS) values for the log linear fit with an R^2 value of 0.98. 30 patient samples were tested in the fluorogenic assay and concentrations were determined using the log linear regression equation and correlated with a standard chromogenic assay used for heparin monitoring.

Results: A statistically significant correlation was found between the fluorogenic and the chromogenic anti-FXa assays for 30 patient samples, with a slope of 0.829, offset of 0.258 and an R^2 value of 0.72 ($p < 0.0001$).

Conclusions: In the study presented here, a fluorogenic anti-FXa assay was correlated with a standard laboratory chromogenic anti-FXa assay using samples from patients on LMWH therapy. Significant correlations between the values derived by the fluorogenic and chromogenic anti-FXa assays were found for the patient cohort tested in this study.

Keywords: AMC; chromogenic; fluorogenic; factor Xa; low molecular weight heparin.

Abbreviations:

7-amino-4-methylcoumarin (AMC)

Activated clotting time (ACT)

Activated partial thromboplastin time (APTT)

Antithrombin (AT)

Factor Xa (FXa)

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

International Normalised Ratio (INR)

Low molecular weight heparin (LMWH)

National Centre for Hereditary Coagulation Disorders (NCHCD)

One way analysis of variance (ANOVA)

Platelet poor plasma (PPP)

Platelet rich plasma (PRP)

Prothrombin time (PT)

SS (Sum of Squares)

TAS-HMT (Thrombolytic Assessment System Heparin Management Test)

Unfractionated heparin (UFH)

Introduction

Low molecular weight heparins (LMWHs) are a group of anticoagulant drugs that are used in the treatment of venous thrombosis, cardiovascular disease, thrombotic and ischaemic stroke worldwide [1, 2]. A significant advantage of LMWHs over unfractionated heparin (UFH) is the fact that monitoring in the large majority of patients is not essential. However, special patient cohorts do exist where monitoring becomes important, and so hospital laboratories must establish suitable methodologies for quantifying the effect of LMWHs [3]. LMWHs undergo renal clearance which can result in anticoagulant accumulation in patients suffering from kidney failure [4, 5]. In addition, dosing of LMWH according to body weight may overestimate the dose for morbidly obese patients, as the anticoagulants may concentrate in the vascular tissue and blood, due to the lower proportion of lean body mass as a percentage of total body weight [4, 6]. Moreover, LMWH monitoring is essential in elderly patients, as lean body mass percentage decreases with age, which can result in overestimation of LMWH dose and in general, bleeding risk also increases with age [4]. Challenges also arise with the prescription of anticoagulants during pregnancy. Changes in maternal weight during the progression of pregnancy, increased bleeding risks in the mother and foetus, and bleeding associated with childbirth all complicate LMWH dosing, reinforcing the need for drug monitoring [6].

Some of the standard coagulation monitoring assays such as the activated partial thromboplastin time test (APTT) or the activated clotting time test (ACT) are not sufficiently discriminatory for monitoring LMWHs, suffer from inter-laboratory variability and are lacking in standardization [2, 7, 8]. LMWHs exert their anticoagulant effect via interaction with the pentasaccharide-binding domain on antithrombin (AT), which in turn enhances the inhibitory effect of AT on factor Xa (FXa) [9]. Due to this high anti-FXa activity of LMWH, anti-FXa assays are the standard monitoring methods for patients on LMWH therapy [5]. The

most widely used central laboratory anti-FXa assays are based on chromogenic substrates [10, 11]. Originally developed by Teien and co-workers in 1976, these assays have a very simple mode of operation whereby heparin-accelerated antithrombin inhibits exogenous FXa and the residual FXa activity is determined by amidolysis of the FXa selective chromogenic peptide substrate. The resultant photometric signal is inversely proportional to the anticoagulant concentration in the sample [12].

A potential advantage of anti-FXa assays is that they are not affected by many of the biological variables that interfere with clot-based endpoints, thus reducing assay variability [13]. Despite improvements in the chromogenic assay mechanism, poor correlative data between commercial anti-FXa chromogenic assays has been reported. This again highlights the need for proper standardization of newly developed assays to reduce inter-laboratory variation and increase confidence in the use of such assays for clinical application.

One major limitation of laboratory-based chromogenic assays is their inability to measure colorimetrically in whole blood samples. Optical clarity is of utmost importance in conducting photometric measurements, rendering turbid media such as whole blood and platelet rich plasma (PRP) unsuitable for application to colorimetric assays [14]. Fluorogenic assays on the other hand, do allow for measurement in whole blood and PRP, as sample opacity is not imperative to the performance of the assay and the fluorescent signal is not hampered by fibrin formation or turbidity caused by platelets [15]. Hence assays using fluorescent labels offer significant advantages over assays using colorimetric determination, in addition to the greater sensitivity and specificity associated with fluorescence measurements [13, 16, 17]. In this paper, we evaluated the suitability of a fluorogenic anti-FXa assay for monitoring LMWH therapy in patient samples using the standard laboratory chromogenic anti-FXa assay as the reference method. Based upon our findings we have

identified a fluorogenic assay for monitoring LMWH therapy, that correlates significantly with the standard chromogenic assay for patients on LMWH therapy ($R^2 = 0.734$, $p < 0.0001$).

Materials and methods

Reagents

Water (molecular biology grade) and HEPES buffer (minimum 99.5% titration) were purchased from Sigma-Aldrich (Dublin, Ireland). Filtered HEPES buffer 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 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). Tinzaparin (Innohep®) was obtained from LEO Pharma (Ballerup, Denmark) and enoxaparin (Clexane®) was obtained from Sanofi-Aventis (Paris, France). 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 stabilize for at least 20 min at room temperature prior to use.

Chromogenic anti-Xa assay and LMWH calibration curve

Chromogenic anti-Xa levels were determined in patient poor plasma using the HemosIL TEST™ HEPARIN chromogenic kit from Instrumentation Laboratory Company (Massachusetts, USA). The kit contained the chromogenic substrate S2765 – N- α -Z-D-Arg-Gly-Arg-pNA.2HCl. This was reconstituted in 4 ml of PCR grade water, left to stabilise at 15-25°C for 30 minutes and inverted before use. Lyophilised purified bovine FXa reagent was dissolved in 5 ml of water, incubated at 15-25°C for 30 minutes and inverted before use.

Antithrombin was diluted in 3 ml of water, left at room temperature for 30 minutes and inverted before use. The stock buffer was diluted 1 in 10 with PCR grade water and from this the working buffer was made by adding 0.5 ml of antithrombin to 12 ml of the stock buffer.

Calibrators of 0, 0.4 and 0.8 U/ml LMWH (Innohep® and Clexane®) were prepared using HemosIL calibration plasma from Instrumentation Laboratory Company (Massachusetts, USA). The 0 and 0.8 U/ml calibrators were diluted 1 in 25 with the working buffer and a 1 in 2 dilution of 0.8 U/ml was performed to generate the 0.4 U/ml calibrator.

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

Fluorogenic anti-Xa assay and LMWH calibration curve

Measurements were carried out in reconstituted citrated human pooled plasma spiked with pharmacologically relevant concentrations (0–1 U/ml) of two low molecular weight heparins; enoxaparin (Clexane®) and tinzaparin (Innohep®). Each well contained 6 µl of 100 mM CaCl₂, 44 µl of pooled plasma and 50 µl of 12 nM FXa. The reaction was started by adding 50 µl of 2.7 µM Pefafuor FXa fluorogenic substrate. These assay concentrations were optimized as previously outlined [13]. All the measurements were carried out in triplicate. 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.

Patient samples

A cohort of 30 frozen plasma samples from patients on LMWH therapy (4 patients on enoxaparin, 16 patients on tinzaparin and 10 unknown LMWH) was collected from the National Centre for Hereditary Coagulation Disorders (NCHCD) at St. James's Hospital, Dublin, Ireland. The anti-FXa levels of all patient samples were determined in the NCHCD, using a HemosIL TEST™ HEPARIN anti-FXa chromogenic assay from Instrumentation Laboratory Company (Massachusetts, USA) for the determination of heparin in plasma, using the ACL 9000™ automated haemostasis testing system also from Instrumentation Laboratory Company. The anti-FXa assay was calibrated by generating calibrators as outlined above under Reagents, with the same LMWH used as therapy, such as Innohep® or Clexane®. The calibration was performed as per the manufacturer's instructions and a calibration run was acceptable when the R^2 value was > 0.98 .

Ethical approval for the use of patient samples was granted by the ethics committee in St. James's Hospital. Patients in this study were on either Innohep® (tinzaparin) or Clexane® (enoxaparin) therapy. All chromogenic anti-FXa levels were determined 3 hours post-administration.

Patient samples were thawed at 37°C in a water bath for 5 min and inverted for 5 min before testing in the anti-FXa fluorogenic assay. The assay protocol was followed as previously described except that calibration plasma was replaced with patient plasma. All measurements were carried out in triplicate.

Data and statistical analysis

In all experiments, reaction progress curves were obtained and analyzed in SigmaPlot 8.0. The reaction rate (slope) was defined as the change in fluorescence divided by the change in time (i.e. dF/dt) and was measured as the linear portion of the fluorescence response profile. Statistical analysis was carried out using SPSS 17.0 software. For statistical analysis, raw

data was transformed logarithmically and analyzed using one-way analysis of variance (ANOVA), with subsequent post-hoc analysis (Duncan, Tukey and Dunnett) if significance was observed. A result of $p < 0.05$ was considered statistically significant.

A single calibration curve of the rate of fluorescence formation versus LMWH concentration was generated by averaging the triplicate measurements for enoxaparin and tinzaparin at each concentration in the sensitive range which was established as 0-1.2 U/ml ($p < 0.0001$). The data for the calibration curve are presented as the mean \pm SD (n=6).

The best fit for the LMWH calibration curve was determined as the linear regression of the log transformation of the data. The reaction rates were calculated for all patient samples and inserted into the linear regression equation to determine the LMWH concentration for each patient sample.

The LMWH concentrations determined by the fluorogenic anti-FXa assay were correlated with the LMWH concentration determined by the chromogenic anti-FXa assay in the NCHCD, St. James's Hospital, Dublin. The HemosIL TEST™ HEPARIN anti-FXa chromogenic assay reports linearity from 0-1.1 U/ml, with run-to-run and day-to-day precision of <10%.

Results

The fluorogenic anti-FXa assay was performed in control plasma samples spiked with increasing concentrations of LMWHs. The dose-response profile was calculated using the initial linear portions of the fluorescence profiles for each LMWH concentration. Linear least squares regression curve fitting was performed on the resulting dose-response curve using log ordinates. Fig. 1 shows the linear regression calibration curve of the log transformation of the raw data reaction rates for calibration plasma spiked with LMWHs from 0 to 1.2 U/ml. The regression equation was $y = -0.713x + 2.081$ and the R^2 value observed was 0.98. Least squares analysis was performed in order to determine the best curve fit for this set of data which returned low sum of squares (SS) values for the log linear fit, indicating small errors and best fit. The fluorogenic assay performance characteristics for LMWH include within run precision CVs of <6.5% (n=6) for all concentrations from 0-1.2 U/ml; the analytical range of the assay was from 0 to 1.2 U/ml; the assay is linear in the 0-1.2 U/ml range with an R^2 value of 0.98 and can measure at intervals of 0.2 U/ml.

To evaluate the fluorogenic anti-FXa assay, 30 plasma samples from patients on LMWH therapy were tested in the assay and the fluorescence profiles of a representative sample of the different profiles can be seen in Fig. 2. All patient samples differed in terms of their fluorescence profiles, which would be expected given the different dosages, time elapsed since dose administration, and clearance rates of the individual patients. Some samples showed rapid increases in fluorescence indicating the presence of low residual drug dosages. Other samples were slow to increase in fluorescence which indicated a high LMWH dose.

The LMWH concentration for these patient samples was also determined using the routine hospital chromogenic anti-FXa assay, so it was possible to correlate the fluorogenic anti-FXa assay with the standard chromogenic assay. The linear reaction rate for each patient was calculated from the fluorescence profiles. Using the fluorescence rate slopes, the LMWH

concentration was calculated using the log linear regression equation, $y = -0.713x + 2.081$. The concentrations were then correlated with the values reported by the hospital chromogenic assay. Fig. 3 shows the correlation between the LMWH concentrations derived from the fluorogenic anti-FXa assay and the chromogenic anti-FXa assay for 30 patient samples which had a slope of 0.829, offset of 0.258 and an R^2 value of 0.72.

Bland-Altman analysis was used to assess the level of agreement between the standard chromogenic assay and the new fluorogenic anti-FXa assay being established. Data were displayed by plotting the difference between the two methods versus the mean of both methods, as can be seen in Fig. 4.

Discussion

LMWHs have been highlighted as more convenient, safe, and effective anticoagulants when compared with UFH [18]. LMWHs have greater bioavailability and are easily absorbed after subcutaneous injection, they have a more predictable dose-response relationship and the lower molecular weight and shorter polysaccharide chain length of LMWHs, results in less non-specific binding to plasma proteins [19]. Despite the general consensus that monitoring for LMWH is unnecessary, patient populations including the elderly, children, pregnant women, patients with renal insufficiency and patients at extreme weights, do exist where dosing of anticoagulants becomes unpredictable [6].

The typical method for monitoring LMWH is by means of the anti-FXa chromogenic assay, which is employed by some central laboratories. Certain drawbacks are associated with chromogenic assays such as lack of standardization and high cost [7]. Sample type is also an issue with these assays in that they can only be performed with platelet poor plasma (PPP) and preparation of PPP requires time-consuming pre-analytical procedures resulting in long turnaround times. Fluorescent detection is a suitable alternative to colorimetric measurement due to high sensitivity and specificity in addition to the broad range of fluorophores and labelling chemistries available for different coagulation proteins [16]. Furthermore, fluorescent detection allows for measurement in PPP in addition to platelet rich plasma (PRP) and whole blood [12, 13]. The aim of the present study was to compare a fluorogenic anti-FXa with the chromogenic anti-FXa assay used in the hospital setting using plasma samples from patients on LMWH therapy.

In this study, a fluorogenic anti-FXa assay is presented that is suitable for measuring LMWH anticoagulants. The fluorogenic anti-FXa assay has a sensitive range up to 1.2 U/ml at intervals of 0.2 U/ml. Plasma samples from patients receiving LMWH therapy were tested in

this assay and correlations were performed with the anti-FXa reference method using linear regression analysis.

In total, 30 patient samples were tested in the fluorogenic assay. Analysis was performed using the log linear regression equation. A significant correlation ($R^2 = 0.72$) between LMWH concentrations derived from the fluorogenic anti-FXa assay and the chromogenic anti-FXa assay was established for patient samples ($p < 0.0001$). The trend observed with the Bland-Altman analysis shows that there is scatter above and below zero, indicating a lack of consistent bias of the chromogenic method over the fluorogenic method. Also apparent is the deviation of the fluorogenic method from the established method with increasing LMWH concentration. Such differences could also be attributable to the difference in the source of FXa in each assay. The FXa used in the chromogenic assay is bovine in origin while the FXa used in the fluorogenic assay is of human origin. This together with the different peptide substrates could be the source of some of the variation between the two assays in the higher heparin range.

The level difference between the routine and novel assays could also be attributed to the different plasmas used in the initial calibration stages of both assays. The calibrations for the chromogenic assay were performed using HemosIL calibration plasma from Instrumentation Laboratory Company (Massachusetts, USA) as the chromogenic kit was also sourced from this company. The fluorogenic assay was previously developed using pooled plasma from Helena Biosciences Europe (Tyne & Wear, UK). This plasma was originally selected, as the HemosIL calibration plasma contains buffers and preservatives that are highly fluorescent at the emission (342 nm) and excitation (440 nm) wavelengths used in the fluorogenic assay. While this plasma may be suitable for use with colorimetric measurements, the use of two different calibration plasmas which contain different constituents could be another source of variability between the two assays.

It should also be noted that two samples lie outside the analytical range of the assay (0-1.2 U/ml). Assay linearity tends to disappear as heparin concentrations increase, however higher concentrations can still be detected. Notwithstanding the inclusion of these, this still resulted in a significant correlation and was again, equally reliable at these elevated concentrations. The same can be said of the chromogenic assay, which is also only specified up to 1 U/ml, but will also give results above this value which are then subject to closer clinical scrutiny. It has long been established that traditional clot-based assays do not return equivalent results for the same patient sample. Variation in reagents, coagulometers, and operators but also in the nature of the mode of assay operation, i.e. clot-based assays, all contribute to the lack of consistency between assay results [20, 21].

The Prothrombin Time (PT) is a clotting assay standardized using the International Normalised Ratio (INR) so as to overcome the problem of assay variability between laboratories. Despite this standardization, poor agreement among PT methods has been observed [22]. Variability in APTT reagent sensitivity for monitoring heparin has also been observed, resulting in a lack of correlation between assays for the same samples [20, 21].

A lack of correlation exists between heparin dose and standard clinical monitoring tests in children on UFH therapy [23]. However, the absence of a correlation may also relate to the fact that assays such as the APTT, have been developed based on adult coagulation systems which are quite different to those of children, in terms of clotting factor levels.

Chromogenic assay comparisons have also resulted in differing results. Kovacs et al. 1999 assessed whether three commercially available chromogenic methods on two different instruments gave equivalent results for patients on anticoagulant therapy. While the R^2 values for the correlations were 0.97-0.99 for UFH and 0.97-0.98 for LMWH, the mean anti-FXa levels were statistically different when analyzed using one-way ANOVA and subsequent Bonferroni analysis. A mean difference of as high as 0.16 U/ml in heparin levels as deduced

by anti-FXa analysis has also been published [24]. Such differences can be attributed to instrument and assay variability. Taking these results into consideration, the authors suggested that the therapeutic heparin range as determined by anti-FXa assays should be instrument and assay specific [24, 25].

Anti-FXa assays vary according to the technique employed [26] as has been outlined by previous correlations between clot-based and chromogenic assays [23, 26]. The comparison of a thrombolytic assessment system heparin management test (TAS HMT) and the ACT with chromogenic anti-FXa levels for 10 patients on heparin therapy, returned statistically significant but marginal correlation coefficients, reported as $R^2 = 0.53$ ($p < 0.002$) and $R^2 = 0.64$ ($p < 0.001$), respectively [27]. To overcome this variability the ideal solution would be the introduction of a global standard assay.

In this study we present fluorogenic anti-FXa assay which has the potential to surpass commercial chromogenic assays in terms of sensitivity, specificity and most importantly in terms of the range of sample types that can be used with this assay. We report a statistically significant correlation between the fluorogenic and the chromogenic anti-FXa assays for thirty patient samples as $R^2 = 0.72$ ($p < 0.0001$) for the log linear calibration. The excellent correlation of both assays observed, indicates the potential of the fluorogenic assay for the laboratory monitoring of LMWH therapy in addition to its inherent advantages as previously described, over colorimetric-based measurement techniques.

In summary, a recently developed fluorogenic anti-FXa assay was correlated with the standard laboratory chromogenic anti-FXa assay using samples from patients on LMWH therapy. Significant correlations between the values derived by the fluorogenic and chromogenic anti-FXa assays were found for the patient cohort tested in this study.

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Figure legends

Fig. 1: Calibration curve for the fluorogenic anti-Factor Xa assay performed according to linear least squares regression of the log transformation of the raw data, $y = -0.809x + 2.106$, $R^2 = 0.995$.

Fig. 2: Fluorescence profiles of a selected number of patient samples in the fluorogenic anti-FXa assay.

Fig. 3: Correlation of calculated concentrations of LMWH activity in 30 patient samples from the fluorogenic and chromogenic assays using the log linear regression fit, $y = 0.758x + 0.230$, $R^2 = 0.734$.

Fig. 4: Bland-Altman plot illustrating differences against averages for the standard chromogenic assay compared with the fluorogenic assay ($y = -0.132x - 0.147$, $R^2 = 0.054$).