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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 (LMWHs) 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 12 nM FXa and 2.7 μM fluorogenic substrate, in addition to 6 μl of 100 mM $CaCl_2$ and 44 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.

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,

Abbreviations: AMC, 7-amino-4-methylcoumarin; ACT, Activated clotting time; aPTT, Activated partial thromboplastin time; AT, Antithrombin; AU, Arbitrary units; CV, Coefficient of variation; FXa, Factor Xa; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LMWH, Low molecular weight heparin; ANOVA, One way analysis of variance; PPP, Platelet poor plasma; PRP, Platelet rich plasma; pNA, *p*-nitroaniline; UFH, Unfractionated heparin.

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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 antithrombin (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 12 nM and 2.7 μM respectively for the fluorogenic anti-FXa assay [20]. Each well contained 6 μl of 100 mM $CaCl_2$, 44 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 measurements were carried out in triplicate.

Commercial chromogenic anti-FXa 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 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 °C for 30 s. Immediately after shaking, absorbance measurements were recorded at 37 °C 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