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Quantification of unfractionated heparin in human plasma and whole blood by means of novel fluorogenic anti-FXa assays

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ABSTRACT

Novel and sensitive plate-based fluorogenic anti-factor Xa (FXa) assays were investigated to quantify unfractionated heparin (UFH) in human plasma and whole blood within the therapeutic ranges of 0–1.6 U/mL and 0–0.8 U/mL, respectively. Two fluorogenic anti-FXa assay methods were defined for low (0–0.6 U/mL) and high (0.6–1.2 U/mL) pharmacologically relevant UFH concentration ranges in pooled human plasma. In both cases significant differences were observed at intervals of 0.2 U/mL ($P < 0.05$). The semi-logarithmic plots of the calibration curves in the low and high UFH range were both fitted to linear regressions with correlation coefficients of 0.96 and >0.99, respectively. The assay was also optimized for whole blood which was capable of differentiating UFH concentrations at intervals of 0.2 U/mL ($P < 0.05$) in the range of 0–0.4 U/mL. The statistically different results were fitted to a linear regression with a correlation coefficient of >0.99. The results obtained in this study could assist diagnostic laboratories towards improved monitoring of UFH therapy.

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1. Introduction

The anticoagulant drugs market is believed to increase to over €9 billion in 2014 from €6 billion in 2008 [1]. Unfractionated heparin (UFH) has been the parenteral anticoagulant of choice for more than 50 years [2–4]. Even though new anticoagulant drugs inhibiting thrombin (30%) and factor Xa (FXa) (70%) are under clinical development [5–7], UFH continues to be administered for short-term prophylaxis because it is effective, inexpensive, and a protamine sulfate antidote exists to rapidly reverse bleeding [8].

Common laboratory monitoring of UFH is carried out by traditional coagulation tests, such as the clot-based activated partial thromboplastin time (APTT) [9], chromogenic anti-FXa assays [10], and the activated clotting time (ACT). Due to some limitations and drawbacks associated with APTT and ACT, it has been recommended to calibrate the therapeutic APTT range in seconds to the reference anti-FXa range of 0.3–0.7 anti-FXa U/mL [11].

Fluorescent detection has been investigated over the last few years as an alternative technique to clotting and chromogenic assays. The high sensitivity that this optical measurement can offer [12] along with its ability to be adapted to a broad range of sample matrices, makes it an interesting technique to investigate. Many fluorophores and labelling chemistries are available for different coagulation proteases such as factor VIIa [13] and factor VIIa-tissue factor complex [14], thrombin [15], factor VIII [16], factor IX aβ [13,17], factor Xa [13,15], factor XIa [18], factor XIIa [19] and factor XIII [20]. In particular, fluorogenic substrates have been used to measure thrombin generation over the last ten years to investigate the effects of UFH in platelet-poor-plasma (PPP) [21] and platelet-rich-plasma (PRP) [22].

To the best of our knowledge, few publications to date have evaluated the use of fluorogenic substrates in the development of anti-FXa assays for monitoring anticoagulant therapy in human plasma [23,24] with none reported for use in whole blood. A novel and sensitive plate-based fluorogenic anti-FXa assay in human plasma was recently investigated to monitor UFH therapy using one of two commercially available peptide substrates for FXa based on a 7-amino-4-methylcoumarin (AMC) fluorescent reporter group

Abbreviations: UFH, unfractionated heparin; FXa, factor Xa; APTT, activated partial thromboplastin time; ACT, activated clotting time; AMC, 7-amino-4-methylcoumarin; SN-7, Mes-D-LGR-ANSN $(C_2H_5)_2$ fluorogenic substrate; ANSN, 6-amino-1-naphthalene-sulfonamide; DCU, Dublin City University; ANOVA, analysis of variance; afu, arbitrary fluorescence units; TEG, thromboelastography; TAS HMT, thrombolytic assessment system heparin management test; TG, thrombin generation.

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[23]. In this study, it was sought to develop novel fluorogenic anti-FXa assays to monitor therapeutic UFH concentrations in both human pooled plasma and whole blood using the only other commercially available FXa fluorogenic substrate.

2. Materials and methods

2.1. Reagents

Water (molecular biology reagent), HEPES (minimum 99.5% titration), sodium citrate tribasic dihydrate (ACS reagent, ≥99.0%) and citric acid monohydrate (ACS reagent, 99.0–102.0%) were purchased from Sigma–Aldrich (Dublin, Ireland). Filtered HEPES (pH 7.4; 10 mM) was prepared and both sodium citrate and citric acid were made up to 0.1 M. Citrate–citric acid buffer solution was prepared at 3.8% sodium citrate and adjusted to ca. pH 5.5 with 0.1 M citric acid. 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 Mes-D-LGR-ANSN $(C_2H_5)_2$ (SN-7), containing the fluorescent reporter group 6-amino-1-naphthalene-sulfonamide (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 aluminum foil. Dilutions of stock solutions were performed with 10 mM HEPES. The kinetic constants of SN-7 fluorogenic substrate, which refer to the cleavage by the endopeptidase FXa, were provided by the supplier as follows: K_m = 125 μM; k_{cat} = 36 s^{-1}; k_{cat}/K_m = 290,000 M^{-1} s^{-1}. Purified human FXa (serine endopeptidase; code number: EC 3.4.21.6) was obtained from Hyphen BioMed (Neuville-Sur-Oise, France). UFH obtained from bovine lung tissue was acquired from Sigma–Aldrich (St. Louis, MO). 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.

2.2. Blood sampling

Human blood was locally obtained from one smoking and five non-smoking healthy volunteers (30–40 years of age; 3 males and 3 females), who had not ingested any pharmacologically active substances prior to the experiment. Informed consent was granted by all volunteers and the study was approved by the Dublin City University (DCU) ethics committee. Samples were drawn through antecubital venipuncture at the School of Health and Human Performance in DCU. 10 mL of venous blood was collected into plastic 20 mL sterile BD Luer-Lok™ syringes from Becton, Dickinson and Company (Drogheda, Ireland), containing 3.8% sodium citrate at a ratio of 1:10 in blood using sterile Venisystems™ Butterfly®-19 needles from Abbott (Sligo, Ireland).

2.3. Apparatus

Absorbance and 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 for fluorescence measurements, and flat, transparent 96-well Greiner® microplates from Greiner Bio-One (Gloucestershire, United Kingdom) for absorbance readings.

2.4. Absorbance and emission spectra of whole blood samples

The total volume of samples in the absorbance and emission fluorescence experiments was 150 μL, which corresponds to the same final volume as used in the fluorogenic anti-FXa assay. Human blood samples were diluted 1:150 in water and their absorption spectra were measured at 2 nm intervals with the Infinite M200 microplate reader from 280 to 1000 nm. Background fluorescence emission spectra were evaluated at the excitation wavelength of 352 nm, which corresponds to that of the ANSN-based fluorogenic substrate, starting at 400 nm up to 850 nm at 2 nm reading intervals. Whole blood was diluted 1:3 in water. All samples were scanned at 37 °C and after the assay was run.

2.5. Optimization of fluorogenic anti-FXa assays

Measurements were carried out in reconstituted citrated human pooled plasma and citrated human whole blood. All assays using human whole blood were initiated within 15 min of collecting the blood samples. The same experimental protocol was used for both matrices, the only difference being the titration range of both FXa and SN-7 fluorogenic substrate. In the presence of citrated human pooled plasma, FXa and the ANSN-based fluorogenic substrate were titrated within the ranges of 0.1–200 nM and 8.3–75 μM (K_m = 125 μM), respectively. In the case of citrated human whole blood, FXa and the fluorogenic substrate were titrated from 300 to 500 nM and from 75 to 125 μM, respectively. The experimental protocol was as follows: samples consisting of 6.25 μL of 100 mM $CaCl_2$, 43.75 μL of pooled plasma or citrated whole blood, and 50 μL of FXa (within the titration ranges) were incubated at 37 °C for 3 min and shaken for the first 150 s. The reaction was started by adding 50 μL of ANSN-based fluorogenic substrate within the titration ranges as previously described. Samples within wells were mixed with the aid of orbital shaking at 37 °C for 30 s. Finally, immediately after shaking, fluorescence measurements were recorded at 37 °C for 60 min with 20 μs integration time. Fluorescence excitation was at 352 nm and emission was monitored at 470 nm, corresponding to the excitation/emission wavelengths of the ANSN fluorophore. All measurements were carried out in triplicate. Following optimization of assay conditions, pooled commercial plasma and whole blood samples were spiked with therapeutically relevant concentrations of UFH from 0 to 1.6 U/mL and from 0 to 0.8 U/mL, respectively. The reaction rate (slope), which is defined as the change in fluorescence divided by the change in time (i.e. dF/dt), was measured as the linear portion of the fluorescence response profile and plotted versus anticoagulant concentration.

2.6. Software and statistical analysis

All graphs were plotted using SigmaPlot 8.0. Statistical analysis was carried out using SPSS 17.0 software. Logarithmic transformation was applied to all reaction rates for data normalization. Intra-assay differences within the anticoagulant concentration range were compared using one-way analysis of variance (ANOVA), with subsequent post-hoc analysis performed (Tukey's test) if significance was observed. A result of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Human plasma

In the first part of the study in plasma, optimization of the fluorogenic anti-FXa assay was undertaken by titrating FXa and the SN-7 fluorogenic substrate within the ranges of 0.1–100 nM and 8.3–33.3 μM (K_m = 125 μM), respectively. The best performing assay was selected in terms of lag time and reaction rate values, as well as an optimal increase in fluorescence response. It was found that 100 nM FXa and 25 μM fluorogenic substrate fulfilled all these performance requirements. Subsequently, the fluorogenic anti-FXa

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