

Article

Optimization of heparin monitoring with anti-FXa assays and impact of dextran sulfate for measuring all in-vivo drug activity

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Abstract:

Heparins, Unfractionated or Low Molecular Weight, are permanently at the spotlight of both clinical indications and laboratory monitoring. An accurate drug dosage is necessary for an efficient and safe therapy. The one-stage anti-FXa kinetics' assays are the most widely and universally used with full automation for large series, without needing exogenous Antithrombin. WHO international standards are available for UFH and LMWH, but external quality assessment surveys still report a high inter-assay variability. This heterogeneity results from: assay formulation, designed without or with dextran sulfate to measure all heparin in blood circulation; calibrators for testing UFH or LMWH with the same curve; and automation parameters. The various factors which impact heparin measurements are reviewed, and we share our experience to optimize assays for completely testing plasma heparin. Evidence is provided on the usefulness of low molecular weight dextran sulfate to mobilize all drug present in blood circulation. Other key factors concern adjustment of assay conditions to obtain fully superimposable calibration curves for UFH and LMWH, and automation parameters. The study is illustrated by the performances of the various anti-FXa assays used for testing heparin on UFH or LMWH treated patients' plasmas and obtained using citrate or CTAD anticoagulants. Comparable results are obtained only when CTAD anticoagulant is used. Using citrate UFH is underestimated in the absence of dextran sulfate. Heparin calibrators, adjustment of automation parameters and data treatment contribute to other smaller differences.

Keywords: heparins; anti-FXa assays; automation; calibration curves superimposition; dextran sulfate

1. Background

Heparin therapy and its monitoring: Heparin and its derivatives, including Unfractionated Heparin (UFH), Low Molecular Weight Heparin (LMWH) and Fondaparinux, remain a major group of anticoagulants with multiple indications in various clinical situations associated with thrombosis or its risk of occurrence [1-3]. Since its discovery, more than 1 century ago [4, 5], and its introduction, heparin is used for preventing or managing thrombotic diseases, and the prognosis of these complications has been totally reversed in many pathological contexts like traumatology, hip or knee replacement, post-surgery, or cardiology [6-9]. A close monitoring is required for therapy adjustment, especially for UFH, to obtain an efficient anticoagulant effect and avoiding bleeding risk [10, 11]. Blood heparin concentration is not always predictable as some patients can present an impaired clearance, especially when there is a deficient kidney function [12, 13], or they can face heparin resistance, in presence of netosis, high circulating histone concentrations or amyloidosis [14-17]. In addition, rare and severe side effects can develop with heparin induced thrombocytopenia, a life-threatening antibody-dependent complication, which requires an immediate heparin withdrawal and use of a different anticoagulant [18, 19]. Heparin remains however the anticoagulant of choice in many critical circumstances, due

to its rapid anticoagulant response and efficacy, but also to its other beneficial activities, anti-inflammatory and antiproliferative [1, 8, 20-22]. Furthermore, heparin can produce blood anticoagulation through additional mechanisms than inhibition of coagulation serine esterases, especially through the release of Tissue Factor Pathway Inhibitor (TFPI) from endothelium, process which is more effective at the onset of therapy, and dependent on heparin sulfation grade and molecular weight [23-2].

The UFH and LMWH drug-dosage needs to be accurately adjusted for each treated patient according to the clinical pattern and physiological status, which can impact drug clearance [2, 6, 13, 26, 27]. If drug concentration is not enough in blood circulation, thrombotic diseases is not correctly controlled, and conversely, if it is too high, due to overdosage or an impaired clearance, patient can bleed. Both situations can lead to a fatal outcome, highlighting the criticism of drug monitoring. Many assays have been developed over time for testing heparin in blood circulation, during open heart surgery, or in plasma [11, 28, 29]. The first assays proposed for evaluating its anticoagulant potential were based on the prolongation of clotting time, and later the activated clotting time (ACT) was introduced for testing high concentrations in cardiology patients, especially in intensive care units [28-30]. However, in clinical settings most of the heparin treated patients have been monitored with the Activated Partial Thromboplastin Time (APTT), performed on citrated plasma, for a long time [31,32]. This clotting method is still the first line laboratory assay in many countries, despite its limitations [33, 34]. The availability of chromogenic assays, introduced about 40 years ago, has permitted the progressive development of more specific methods for testing heparin concentrations in plasma [10, 30, 33, 35-38]. Specific thrombin or FXa chromogenic substrates are used for enzyme inhibition methods. This lead to develop first 2-stage assays, then anti-FXa kinetics assays, fully automated. These latter are now the most widely used with the various available coagulation instruments. Heparin measurements are much more accurate when monitored with chromogenic assays than when tested with APTT or ACT [33, 35, 38], as these clotting methods present many interferences, especially in severely ill patients. They can result from high Factor VIII concentration [31], inappropriate citrate content in blood samples obtained in insufficiently filled tubes, blood activation during collection, or low hematocrit.

Mode of action of heparin: Heparin is an indirect catalytic inhibitor and requires Anti-Thrombin (AT) for inhibiting coagulation serine esterases, mainly thrombin, also named activated Factor II (FIIa), and FXa, and in a lesser extend FIXa, FXIa and FVIIa [39, 40]. In the absence of heparin, AT is a progressive inhibitor of thrombin and FXa. When present, heparin binds to AT through an irregular pentasaccharide sequence, in a molecule-to-molecule complex. AT becomes then a fast-acting inhibitor of thrombin and FXa and forms finally a stable irreversible complex with these serine esterases, whilst heparin is released from the complex and becomes available for activating a new AT molecule [41, 42]. The limiting factor for the anticoagulant action of heparin, in addition to its concentration, is then the concentration of AT, and the drug turn-over for inhibiting serine esterases. The turn-over of heparin for AT activation, and therefore its anticoagulant potential, depends on its characteristics, especially the pentasaccharide sequences' density and its molecular weight (MW) or polysaccharide length [4, 5, 43]. In body or in the assay system, heparin anticoagulant activity is dependent on AT concentration only if that one is too low. When AT is present at an enough concentration, anticoagulant activity is then heparin dose dependent. Other characteristics of heparin, like the global electronegative charge and the sulfate groups density, affect better its non-anticoagulant biological effects [8, 9, 21, 2]. Heparin is an electronegatively charged molecule which can interact with many blood proteins and bind to various blood cells through exposed surface proteins, especially to endothelial cells and platelets [44, 45]. UFH has a higher affinity for blood proteins and cells than LMWH. Proteins which can impact heparin activity in blood or

plasma are first platelet factor 4 (PF4), a protein released from platelet α -granules and which has the highest affinity and can neutralize this drug at stoichiometric concentrations [4], then Histidine-Rich-Glyco-Protein (HRGP), a protein involved in fibrinolysis for the regulation of plasminogen binding to fibrin [47, 48]. But other proteins can also bind to heparin with a lower affinity, like vitronectin, β 2-Glycoprotein, but their incidence on heparin activity is negligible.

Chromogenic assays for heparin monitoring: The first heparin chromogenic assays introduced were the 2-stage assays, based on the inhibition of a constant amount of FIIa or FXa. Diluted tested specimen is mixed with a constant concentration of purified AT and FIIa or FXa for a fixed time, in a first step, followed by the addition of the chromogenic substrate, which reacts with the non-inhibited FIIa or FXa, in the second step [49, 50]. An inverse dose-response curve is obtained between heparin concentrations and absorbance, measured at 405 nm. The assays must be calibrated with the same type of heparin measured, in a like-to-like manner. Calibrators are prepared by spiking the assayed drug in normal citrated plasma or in the assay buffer for obtaining the reference range. Performing these laboratory methods requires a high level of technical expertise, and the assay conditions need to be strictly adhered to. Each stage is critical, and the timing must be respected exactly. High quality biochemicals, including AT, FIIa or FXa, and chromogenic substrates, are required. These assays are extremely sensitive, with ranges from ≤ 0.10 IU/mL for anti-FXa or ≤ 0.05 IU/ml for anti-FIIa methods. Samples containing heparin must be highly diluted before testing. When heparin is assayed in plasma, a platelet depleted plasma with a low PF4 content (< 10 ng/mL) is required for preparing calibrators. For the value assignment of heparin drugs, a reference range is prepared in the assay buffer containing Bovine Serum Albumin (BSA) or Poly-Ethylene-Glycol (PEG 6000) as carrier substances. The exact conditions for performing these assays are documented in Pharmacopeias (EP, USP, JP). These assays' constraints have limited the use and automation for these methods, especially since the introduction of automated instruments, which face limitations for managing exactly the 2 exact incubation times required. The 2-stage assays however remain the reference methods for testing heparin and its derivatives by pharmaceutical industry in association with like-to-like drug reference materials [51].

Automated one-stage anti-FXa kinetics methods have been developed for the current laboratory monitoring of heparin therapy, along with plasma calibrators for UFH, LMWH or Fondaparinux. These assays can be automated on any of the coagulation instruments now available in laboratories and an assay precalibration is currently used [30, 52]. A new calibration is only required from time to time, the permanence of measurement performances being verified daily with control plasmas. No exogenous AT is needed for kinetics anti-FXa assays, and endogenous assayed plasma AT is enough in when $\geq 50\%$. Cautions are required for testing plasmas from pediatric patients, or from patients with a low AT ($< 50\%$). For performing the assay, the tested plasma, undiluted or slightly diluted with physiological saline or assay buffer, is automatically pipetted into the instrument reactive cuvette, and is mixed at 37°C with the FXa specific chromogenic substrate at an optimized concentration; when the temperature is equilibrated at 37°C , 1 to 2 minutes later, a constant and in excess concentration of FXa, prewarmed at 37°C , is added and the reaction starts. There is a competition of FXa for the AT-heparin complexes and its chromogenic substrate. Higher is the heparin concentration and lesser FXa is available for cleaving the substrate. The change in absorbance, measured at 405 nm, is an indirect relationship of heparin concentration. The assay calibrator is obtained with heparin spiked in plasma at various concentrations, covering the dynamic range. WHO International Standards (IS) are available for UFH and LMWH and allow standardization and traceability of calibrators proposed by each heparin diagnostic device manufacturer [53, 54]. As each heparin type has a specific inhibition kinetics for FXa, plasma calibrators

prepared with the same heparin tested must be used. However, there is a strong market request to use a single heparin calibration for all heparin types, whether UFH or LMWH. Most manufacturers now propose a single heparin calibration curve, hybrid, for testing all heparins. This goal is achieved correctly fully superimposable UFH and LMWH calibration are obtained. Today, the current practice for monitoring any type of heparin therapy is to use the one-stage anti-FXa chromogenic kinetics assay, fully automated, with only one precalibrated curve, associated with UFH or LMWH control plasmas.

Variability of heparin measurements: Although important efforts have been performed to standardize, automate, and optimize heparin testing, with availability of ISs and of guidelines issued by scientific societies or regulatory bodies, many differences in measured plasma heparin concentrations are still observed when the various branded heparin anti-FXa chromogenic assays are used [55-58]. This is illustrated by the external quality assessment programs, like ECAT, which show a remaining significant reagent to reagent and laboratory to laboratory variability, more especially for UFH in the low range [57]. The debate on which anti-FXa method generates the right results has been recently open again, with the extended indications of heparin treatments, using either UFH or LMWH, in Covid-19 patients, as thrombosis is a frequent disease complication [55, 59-61].

Indeed, heparin measurement is an assay which concerns a catalytic indirect inhibitor, and many parameters impact its kinetics. The design of assay conditions is essential for its performances. With the same assay principle, the presence of multiple proteins binding to heparin in plasma produce significant differences depending on the reagent concept, its formulation and the calibration used. Many years ago, the use of low molecular weight dextran sulfate (DS) was introduced for improving the heparin anti-FXa assays. It was claimed that presence of this component allowed measuring the full heparin activity in plasma, by limiting the impact of ex-vivo neutralization, especially by platelet released products [62-64]. Now, many heparin diagnostic device manufacturers use this component, which is indicated on the instructions for use, whilst others do not yet [57,61].

Another important incidence on measured heparin concentrations results from the calibration used. Heparin can be often tested in emergency conditions. Clinical laboratories do not always know which heparin brand or type is used for patients' treatments. There is then a high expectation to use a single heparin calibration for any heparin type to be measured. Attempts have been done for reaching this objective. One approach is to develop assay conditions, which permit obtaining the same dose-response curve for UFH and LMWH [62, 63]. Calibration curves for UFH and LMWH are then fully superimposable. Another approach is to build a hybrid curve by mixing or combining UFH and LMWH for plasma calibrators to get a median curve, between that of UFH and that of LMWH [65]. In this report we show the impact of DS for measuring the various heparin types, and its contribution to the exactness and accuracy of heparin measurement on plasma. Reagents and reference material from the various manufacturers are compared for the measurement of UFH or LMWH on citrate or CTAD anticoagulated plasmas from heparin treated patients [66]. Assays are calibrated with the manufacturers' proposed heparin calibrators comparatively to the WHO UFH or LMWH International Standards. We then discuss the factors which are responsible for the variations of measured heparin concentrations and the assays' biases.

2. Materials and Methods

Patients and normal plasmas: citrated normal plasmas and plasma pool were supplied frozen by Precision Biologic Inc. (Halifax, Canada), and stored at $< -70^{\circ}\text{C}$ until use.

Plasmas from hospitalized patients with heparin therapy for post-surgery thrombosis prevention, using either UFH or LMWH, were obtained from Beaujon University Hospital (Clichy, France), as the left-over residual plasma, and obtained according to CLSI. Blood was collected either on 0.109 citrate or CTAD (Citrate-Theophylline-Adenosine-Dipyridamole) anticoagulant from heparin treated patients (UFH or LMWH), and plasma was decanted following 20 minutes centrifugation at 2,000 g, at Room Temperature (RT), then stored frozen at -70°C until use. Plasmas were thawed for 5 min in a water bath at 37°C just before use.

Heparin anti-FXa kinetics chromogenic assays were obtained from various manufacturers: STA-Liquid Anti-Xa (reagent A), and STA-Multi-Hep Calibrator, STA-Quality HNF/UFH and STA-Quality HBPM/LMWH from Diagnostica Stago (Asnières, France); HemosIL Liquid Anti-Xa (reagent B), HemosIL Heparin Calibrators, HemosIL UF Heparin Controls and HemosIL LMW Heparin Controls from IL-Werfen (Le Pré Saint Gervais, France); INNOVANCE Heparin (reagent C), INNOVANCE Heparin Calibrator, INNOVANCE Heparin UF Controls and INNOVANCE Heparin LMW Controls from Siemens (Aubervilliers, France); BIOPHEN Heparin LRT (reagent D), the 2-stage assays, BIOPHEN Heparin Anti-Xa-2-stages and BIOPHEN Heparin Anti-IIa-2-stages, BIOPHEN UFH calibrator and controls, and BIOPHEN LMWH calibrator and controls, were from HYPHEN BioMed (Neuville sur Oise, France). IL-Werfen and Diagnostica Stago propose plasma calibrators prepared by mixing or combining UFH and LMWH with traceability to International Standards and claim a hybrid calibration curve which can be used irrelevantly for UFH or LMWH. Siemens propose plasma calibrators prepared with LMWH only, and Hyphen BioMed propose a full superimposition of UFH and LMWH calibration curves, whether UFH or LMWH plasma calibrator is used; in practice plasma calibrators are prepared with LMWH. Siemens, IL-Werfen and HYPHEN BioMed anti-Xa reagents (B, C and D) contain dextran sulfate (DS), whilst the Diagnostica Stago one (A) does not.

Reference materials used for UFH or LMWH were the WHO International Standards (IS), obtained from the National Institute for Biological Standards and Controls (NIBSC, Pottery Bar, UK): IS 11/176 for LMWH (1068 anti-FXa and 342 anti-FIIa IU per ampoule) and IS 07/328 for UFH (2,145 IU per ampoule). These ISs were restored as indicated on the product instructions for use, and a stock solution was prepared at exactly 100 International Units (IU)/mL using a 0.05 M Tris, 0.15 M NaCl, 1% BSA buffer at pH 7.40 (TBSA). This stock solution was used for preparing UFH or LMWH concentration ranges in the Cryocheck plasma pool, from 0 to 1.8 IU/mL: first a twenty-fold concentrated range was prepared in TBSA (0 to 36 IU/mL); then 50 μL of each stock solution was spiked in 950 μL of cryocheck citrate plasma pool to obtain an UFH or LMWH concentration in plasma ranging from 0.00 to 1.80 IU/mL. All spiked plasmas had the same matrix, i.e. 95% cryocheck plasma pool and 5% TBSA.

Laboratory coagulation automated instruments: each heparin anti-FXa assay was used with the manufacturer's proposed instrument: Diagnostica Stago reagent A with STA-R Max (Diagnostica Stago, Asnières, France); IL-Werfen reagent B with ACL-Top 550 (IL Werfen, Le Pré St Gervais, France); Siemens reagent C with CS-5100 (an automated instrument from Sysmex, Kobe, Japan, and distributed by Siemens Healthineers, Aubervilliers, France); HYPHEN BioMed reagent D with the Sysmex CS-5100 instrument (Sysmex, Kobe, Japan); the BIOPHEN 2-stage anti-FIIa and anti-Xa assays were used with the CS-2400 instrument (Sysmex, Kobe, Japan). Reagents were used respecting strictly the manufacturers' protocols recommended. HYPHEN BioMed reagents, which are multi-platform, were used with the CE marked protocols developed and validated for CS-5100. All tested plasmas were used undiluted (reagent B) or diluted as claimed in the instruc-

tions for use for each assay, and plasma diluent was either Owren Veronal Buffer (reagents A and C), or 0.15 M sodium chloride (reagents C and D).

Verification of dose-response curves for UFH and LMWH: the citrate plasma pool supplemented with either UFH or LMWH ISs was assayed for each reagent-instrument combination (A, B, C and D), parallelly with the manufacturers' calibrators.

Correlation studies: all plasmas, from UFH or LMWH treated patients, and whether citrate or CTAD anticoagulated, were tested with the 4 anti-FXa assay combinations and correlation diagrams were established. Sub-analysis was then performed for the various groups, plasmas from UFH or LMWH treated patients, obtained using citrate or CTAD anticoagulant.

Heparin characteristics of plasma calibrators: heparin calibrators from the various manufacturers were tested with the 2-stage anti-FXa or anti-FIIa assays with the CS-2400 instrument and calibrated with the UFH or LMWH WHO-ISs spiked in plasma. This measurement allowed analyzing the content of each plasma calibrator by establishing the anti-FXa/Anti-FIIa ratios: UFH has a ratio of 1.00, whilst depending on the branded material LMWH has a ratio from 1.6 to 9.7 [4, 43].

Calibration curves analysis: Heparin calibrators proposed by each manufacturer for its anti-FXa kinetics assay were evaluated comparatively to UFH and LMWH WHO-ISs. Each proposed manufacturer's heparin calibrator and the UFH or LMWH WHO-ISs spiked in plasma, with a concentration range from 0.00 to 1.80 IU/ml, as described before, were tested with each anti-FXa reagent-instrument combination, as described here above (A, B, C and D). For each combination, the 3 calibration curves obtained (heparin assay manufacturer's calibrator, UFH IS and LMWH IS) are compared.

Statistics were performed using the analyse-it software.

3. Results

Calibration curves for the various assays:

The various calibration curves obtained with each anti-FXa combination for the manufacturer's calibrator and the UFH or LMWH WHO-ISs are shown on figure 1. Superimposition between the manufacturer calibration curve and those obtained with the WHO International UFH or LMWH Standards is globally good, although some slight deviation can be seen depending on the system used. In combination A, UFH-IS calibration lacks linearity, especially in the low range, and absorbances measured are above the manufacturers' calibration, which can result in underestimation of UFH concentrations, especially for low heparin concentrations. Superimposition is better in the high range. In combination B, UFH and LMWH ISs calibrations have an acceptable superimposition, and manufacturer's calibration appears to deviate below ISs curves, which can underestimate UFH or LMWH concentrations. In combination C, superimposition is also acceptable, with the assay calibration like that of UFH-IS but slightly above that of LMWH-IS, which can tend to slightly underestimate LMWH; superimposition for all the curves is also obtained for combination D.

Deviations are higher for UFH, especially for low concentrations, when DS is not used in the assay system. A better accuracy and exactness are also obtained when heparin plasma calibrator concentrations are regularly distributed over the dynamic range, than concentrated in the lower part, as for combination B.

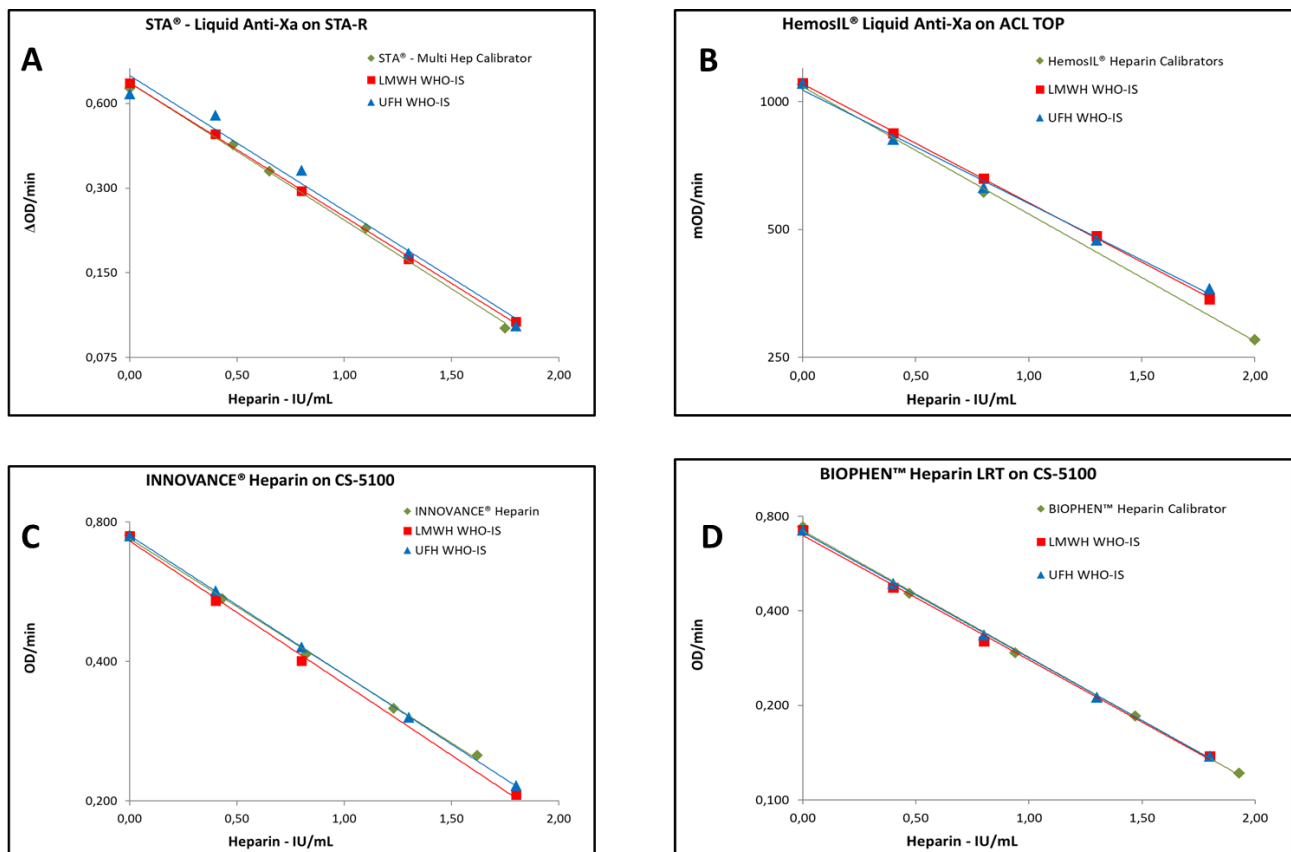


Figure 1. Comparison of calibration curves for each anti-FXa assay used with the manufacturer's coagulation instrument as compared to the International Standards for UFH or LMWH. Heparin concentrations are on abscissae and change in absorbance per minute (OD/min) on ordinates.

Correlation studies:

Correlation studies are performed on the global patients' plasma group, obtained from blood samples from UFH or LMWH treated patients and collected on citrate or CTAD anticoagulants. Figure 2 shows the various correlation diagrams, for each manufacturer's device compared to the others: B vs D; C vs D; C vs B; D vs A; C vs A; B vs A. The reagents containing DS (B, C and D) present acceptable correlations between them, whilst there is a higher dispersion when these reagents are compared with reagent A, designed without DS.

The differences are higher for UFH samples than for LMWH. The correlation line tendency for A and B is to underestimate heparin concentrations as compared to C and D, as expected from the calibration curve analysis.

The mean values for the various subgroups of plasmas tested (UFH or LMWH with citrate anticoagulant or with CTAD anticoagulant) are shown on table 1. Mean heparin concentrations are lower when measured with reagents A and B than with reagents C and D. Differences are partly due to the use of dextran sulfate for the assay formulation, and partly to the calibration used.

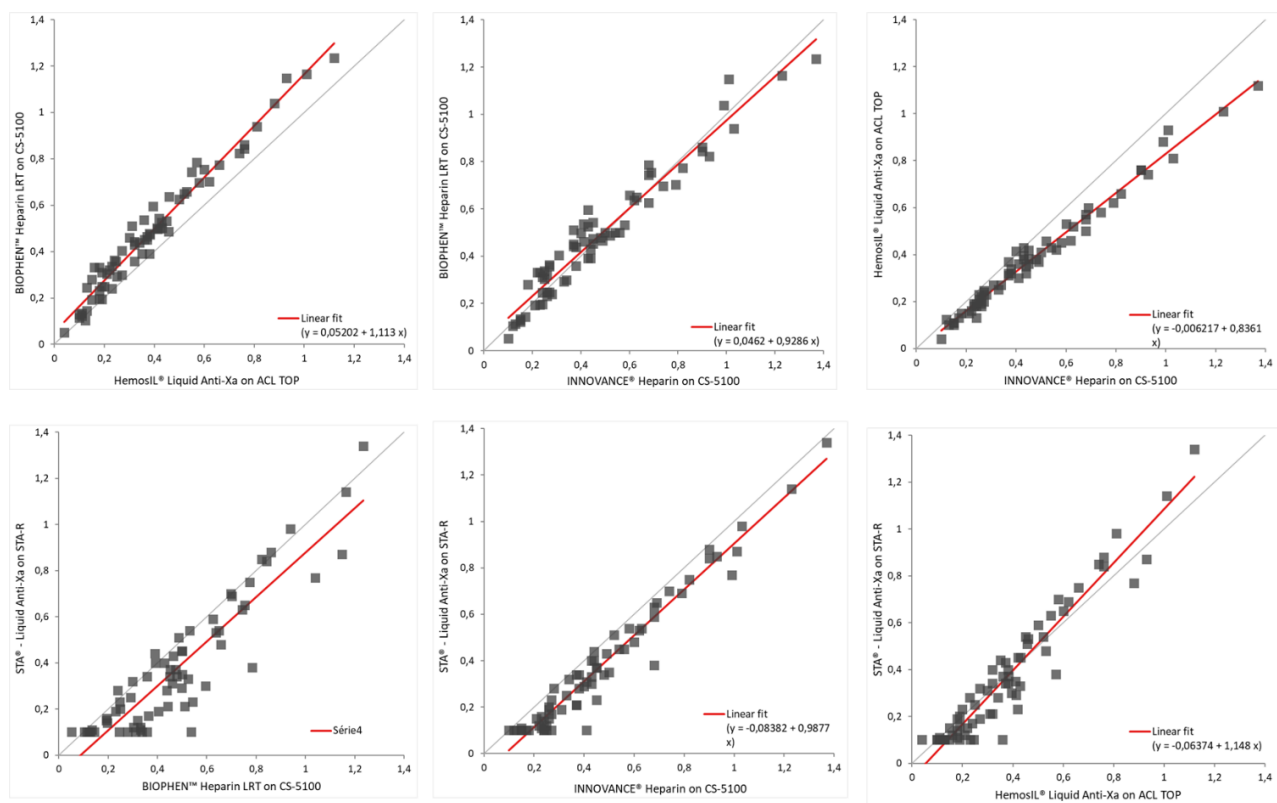


Figure 2. shows the global cross correlations between the 4 different branded anti-FXa assays for all the tested plasma samples from heparin treated patients (either with UFH or LMWH), and anticoagulated with citrate or CTAD. Assays B, C and D contain dextran sulfate, whilst assay A does not. The global correlation is better when assays containing dextran sulfate are compared between them.

Table 1: Mean heparin concentrations measured on the 68 plasmas from UFH or LMWH treated patients, using the 4 various anti-FXa kinetics assays (Stago, Werfen-IL, Siemens and HYPHEN BioMed); blood was collected either on citrate or CTAD anticoagulant, and plasma decanted following centrifugation.

N=68	Mean	SD	Minimum	Median	Maximum
STA® - Liquid Anti-Xa (A)	0.376	0.282	0.10	0.315	1.34
HemosIL® Liquid Anti-Xa (B)	0.383	0.235	0.04	0.355	1.12
INNOVANCE® Heparin (C)	0.466	0.278	0.10	0.410	1.37
BIOPHEN™ Heparin LRT (D)	0.479	0.266	0.05	0.452	1.24

Impact of anticoagulant:

To understand and illustrate which factors are responsible for heparin concentration differences between assays, the various correlation diagrams were drawn by identifying each patients' plasma group. Figure 3 shows for each combination the correlation diagrams with the separate identification of each subgroup: UFH-Citrate; LMWH-Citrate; UFH-CTAD; LMWH-CTAD. This diagram shows obviously that the differences are mainly due to citrate plasma samples containing UFH, and in a lesser extend to citrate samples containing LMWH. When CTAD is used as anticoagulant, a much better coherence of heparin concentrations measured is obtained between all assays.

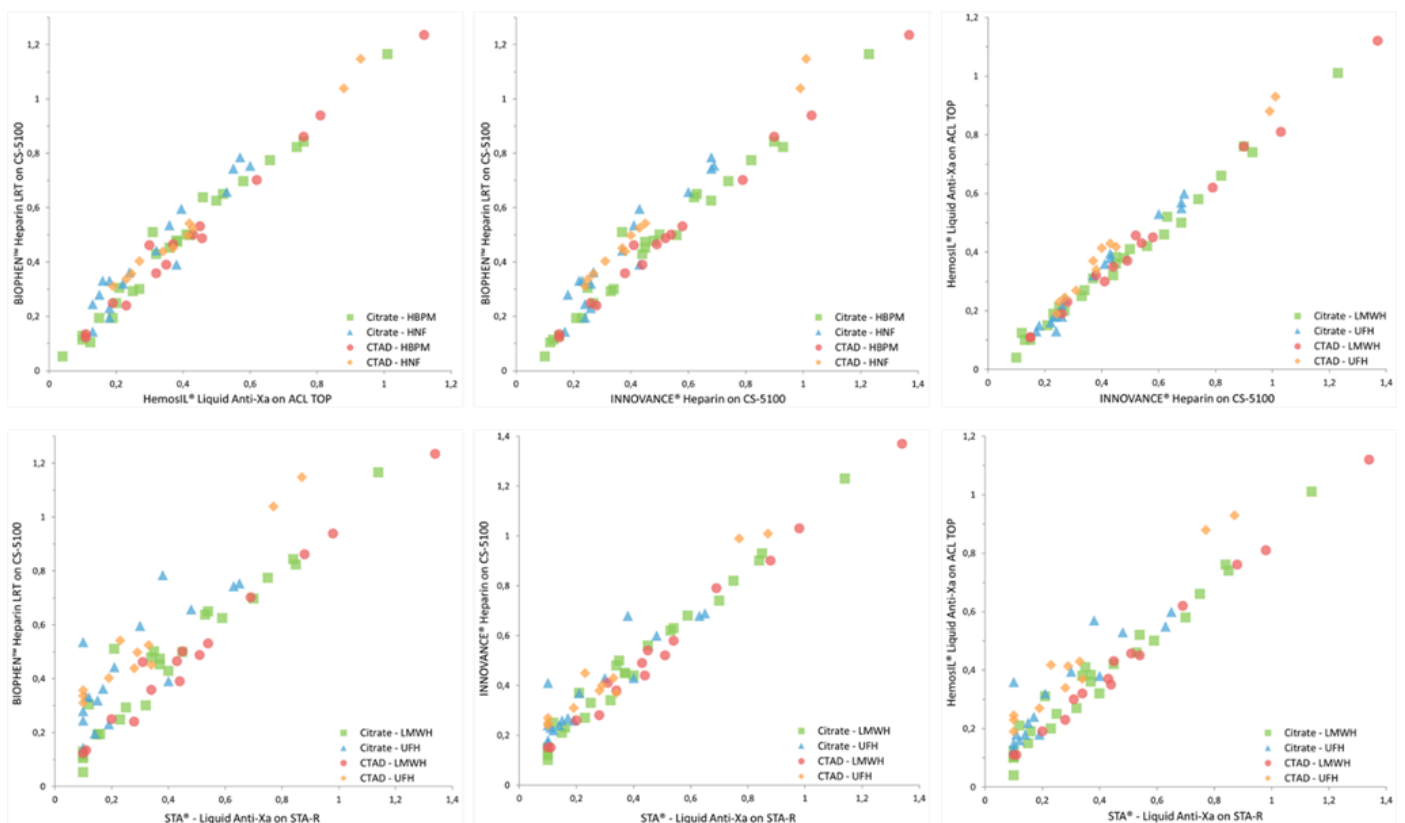


Figure 3. Crossed correlations for the comparison of the various tested subgroups (UFH-Citrate: blue triangles; LMWH-Citrate: green squares; UFH-CTAD: orange dots; LMWH-citrate: orange diamonds) with the various reagent-instrument combinations A, B, C and D. The highest differences are observed for UFH-citrate, and in a lesser extend for LMWH-citrate, plasma samples, especially when anti-FXa reagents with (B, C and D) or without (A) dextran sulfate are compared.

To confirm the factors explaining the heparin concentration differences measured with the various reagents, especially when designed with or without DS, correlations were analyzed separately for each group of plasma samples, obtained from blood collected on citrate or CTAD anticoagulant as shown on the correlation diagrams presented on figure 4. Results are shown for UFH or LMWH plasmas with the 2 anticoagulants, citrate or CTAD, only for the comparison between reagents A and D. However, similar correlations are obtained for A when compared to reagents B or C (data not shown).

The highest dispersion of results between reagents A and D concerns UFH samples collected on citrate anticoagulant. When the same samples are collected on CTAD anticoagulant a much better correlation is obtained. The same comments can be done when comparison is made between reagent A and reagents B or C, whilst correlations are ac-

ceptable when reagents B, C and D are compared between whether on UFH and LMWH plasmas, anticoagulated with citrate or CTAD.

These data suggest that UFH is partially inhibited ex-vivo and its concentration is underestimated when reagent A is used. Presence of DS prevents from this inhibition.

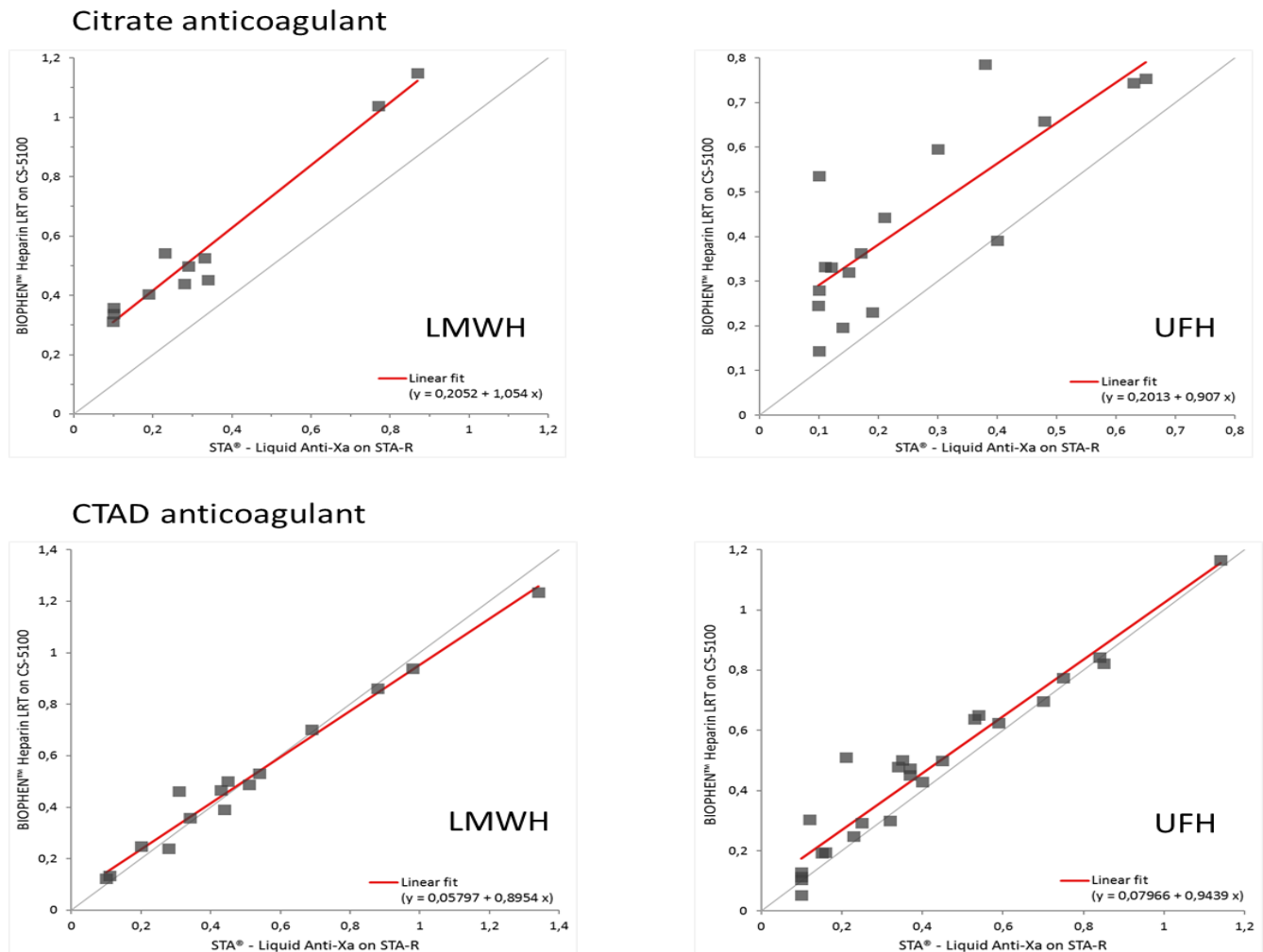


Figure 4. correlation diagrams between the anti-FXa reagent designed without dextran sulfate (A) and another one with (D) for the various subgroups of tested samples: citrate-UFH; citrate-LMWH; CTAD-UFH; CTAD-LMWH. The correlation is poor for citrate anticoagulated samples, especially for UFH, whilst it is acceptable for CTAD anticoagulated plasmas, containing either UFH or LMWH.

The mean heparin concentrations measured with the 4 anti-FXa assays combinations were analyzed for each of the subgroups treated with either UFH or LMWH, and anticoagulated with citrate or CTAD. Table 2 shows the values obtained for each subgroup, underlining the important impact of the anticoagulant used and assay design without DS, on the heparin concentrations measured especially for the low concentration range. Other differences observed with the various assays and the various groups can be explained by the calibration curves biases, when compared with the UFH or LMWH reference curves obtained with the ISs. This has an additional impact on reagent B in the low UFH range, and in a lesser extent on reagent C.

Table 2: mean heparin concentrations, in IU/mL, measured with the 4 different anti-FXa reagents on the various subgroups: Citrate-UFH; Citrate-LMWH; CTAD-UFH and CTAD-LMWH).

			STA® LIQUID ANTI-XA	HEMOSIL® LIQUID ANTI-XA	INNOVANCE® HEPARIN	BIOPHEN™ HEPARIN LRT
UFH (IU/ML)	Citrate	Mean	0.25	0.31	0.37	0.43
	N=17	Min-Max	0.10-0.65	0.13-0.60	0.17-0.69	0.14-0.79
	CTAD	Mean	0.33	0.43	0.46	0.55
	N=11	Min-Max	0.10-0.87	0.19-0.93	0.24-1.01	0.31-1.15
LMWH (IU/ML)	Citrate	Mean	0.40	0.38	0.48	0.46
	N=25	Min-Max	0.10-1.14	0.04-1.01	0.10-1.23	0.05-1.17
	CTAD	Mean	0.51	0.44	0.55	0.51
	N=15	Min-Max	0.10-1.34	0.11-1.12	0.15-1.37	0.12-1.24

Composition of the various heparin calibrators:

As heparin anti-FXa reagents are indicated for testing all heparin types, manufacturers proposed superimposed curves or hybrid curves which can be used irrelevantly for testing UFH or LMWH with the same heparin calibrator. We evaluated the specific anticoagulant activity of each heparin plasma calibrator to FXa and FIIa, with the 2-stages assays. The specific anti-FXa to anti-FIIa ratios were calculated for each calibrator. Results are presented on table 1.

UFH has an anti-FXa/Anti-FIIa ratio of 1.00 and the various LMWH have ratios ranging from 1.6 to 9.7, partly dependent on the MW size distribution, and on the pentasaccharide density. From these data it can be deduced that Stago heparin calibrator set contains 2 calibrators (calibrators 2 and 4) obtained by supplementing plasma with UFH and 2 with LMWH (calibrators 3 and 5), whilst all the IL HemosIL heparin calibrators contain a mixture of UFH with some LMWH. Siemens and HYPHEN BioMed heparin calibrators are homogenous and prepared with only LMWH added to plasma. The anti-FXa to Anti-FIIa ratios show that different LMWH are used: this ratio (mean of 2.10) is lower for the Siemens calibrators, like that of certoparin, and higher for HYPHEN BioMed (mean of 4.02), like that of enoxaparin. The WHO International Standard for LMWH 11/176 has an Anti-FXa/FIIa ratio of 3.12 (1068 IU for anti-FXa and 342 IU for anti-FIIa).

The appropriateness for the use of a single heparin calibration curve for measuring UFH or LMWH depends first on the accuracy of the superimposition of both curves obtained with the corresponding ISs. Both WHO standards were proposed as each heparin type, UFH or LMWH, present different characteristics for inhibition kinetics.

Table3: Analysis of the various heparin calibrators from the different manufacturers by testing their anti-FXa and anti-FIIa activities (IU/mL) as compared with the manufacturers' claimed concentrations for the used heparin calibrator from the lots used, and anti-FXa/anti-FIIa ratios.

Brand	Heparin Calibrators	Anti-IIa IU/ml	Anti-FXa IU/ml	Anti-FXa/Anti-IIa ratio	Manufacturer's Target Value IU/ml
IL	1	0.00	0.02	0.00	0.00
	2	0.59	0.77	1.32	0.80
	3	1.68	2.32	1.38	2.00
Diagnostica Stago	1	0.00	0.02	0.00	0.00
	2	0.59	0.57	0.98	0.40
	3	0.38	0.82	2.08	0.68
	4	1.12	1.08	0.95	0.98
	5	1.06	2.18	2.06	1.79
Siemens	1	0.00	0.00	0.00	0.00
	2	0.18	0.37	2.06	0.43
	3	0.38	0.79	2.08	0.85
	4	0.56	1.19	2.13	1.27
	5	0.74	1.58	2.14	1.67
HYPHEN BioMed	1	0.00	0.00	0.00	0.00
	2	0.11	0.44	4.00	0.45
	3	0.23	0.92	4.00	0.92
	4	0.33	1.35	4.09	1.36
	5	0.48	1.92	4.00	1.80

4. Discussion

Recent articles have pointed out the variability of heparin measurements using the various commercially available Anti-FXa assays. This debate has been reactivated with the extended use of heparin therapy in Covid-19 patients, and the detection in some patients of high sensitivity, when drug clearance is decreased, or resistance, when strong inflammation, Nets and histones are present (14, 15, 67, 68). Some recent studies suggest that there is an overestimation of measured heparin concentrations, especially for UFH, when DS is used for the anti-FXa assay formulation, whilst other reports support this technical choice as providing the most accurate estimation of circulating heparin anticoagulant activity [55, 59, 60]. Especially, this debate questions which is the right residual heparin concentration following neutralization with protamine sulfate at the end of extra-corporeal circulation, and when the rebound effect is observed [59, 69-71]. Studies using heparinase or heparinase showed that the measured residual heparin does not always match with the anticoagulant activity measured [72-74], and presence of DS can provide a better estimation. As a developer of heparin testing reagents, we analyzed these different reports and anti-FXa assays' performances through our experience.

In this study we have evaluated various factors impacting the measurement of heparin concentrations on plasma from UFH or LMWH treated patients using the 4 major commercially available anti-FXa assays. We have investigated the incidence of assays' formulations, and of the manufacturers' calibration curves proposed. Heparin calibrators have been tested by comparison with the UFH or LMWH WHO International Standards, spiked in a normal platelet poor plasma pool. Three of the anti-FXa assays (reagents B/IL-Werfen, C/Siemens-Innovance and D/ HYPHEN BioMed-Biophen) are designed with dextran sulfate, a component which was reported to make available for testing all mobilizable heparin with anticoagulant activity, as present in the sample [62, 63], whilst

one (reagent A/Stago-STA Liquid Anti-Xa) does not contain this component. Tested plasma samples were provided by Beaujon University' Hospital (Clichy, France) and were obtained from UFH or LMWH treated patients, collected on citrate or on CTAD anticoagulant. CTAD tends to be less and less used nowadays, as laboratories wish to use only 1 anticoagulant tube type for standardizing hemostasis testing. CTAD was developed to increase blood and plasma stability, especially for heparin testing [66, 75]. This anticoagulant formulation prevents from platelet activation and release of heparin neutralization proteins. The assay-to-assay comparison was performed on the global group including 68 plasmas and analyzed for each subgroup concerning the heparin type and the anticoagulant used. Four groups were then obtained: UFH-citrate (N=17); LMWH-citrate (N=25); UFH-CTAD (N=11); LMWH-CTAD (N=15). Globally, on the total group, correlations were acceptable between reagents B, C and D, and there was a higher dispersion of values and a poorer correlation between reagent A and the 3 others, especially in the low range. When subgroups were analyzed separately, it was obvious that major deviations were observed for plasmas from UFH treated patients and anticoagulated with citrate. A better homogeneity was obtained when samples were collected using CTAD anticoagulant, thus preventing from heparin neutralization ex-vivo. Correlations between assays are much better when plasma samples are collected on CTAD. These results strongly suggest that UFH is partly neutralized ex-vivo by platelet released proteins, and that this inhibition is prevented using CTAD anticoagulant. When reagents contain DS, this inhibition is prevented, and heparin concentrations measured are expected to match better with those present in blood circulation, and this observation is supported by various studies [60, 62, 63, 74].

When comparing the mean heparin concentrations and standard deviations, the lowest values were obtained with reagent A, then B, especially for UFH, and the highest ones with reagents C, then D. Despite reagents B, C and D all contain DS in their formulation, some differences were observed between the mean concentrations measured, especially for reagent B as compared to reagents C and D. However, SDs are similar between reagents. The choice of the calibration curve for measuring irrelevantly plasma samples containing UFH or LMWH contributes to explain these differences. Clinical laboratories need a 24/24 h and 7/7 d available anti-FXa assay for measuring heparin and monitoring treated patients, and some of the analysis are requested in emergency. The type of heparin used is not always known, and therefore using a single calibration curve is necessary. This approach needs to be carefully established and validated to give accurate results for all types of heparin used. WHO proposes nevertheless 2 separate International Standards for UFH or LMWH, and assay manufacturers need to establish anti-FXa assay conditions to obtain fully superimposable calibration curves for all heparin types. When the right conditions are fulfilled, calibration curves obtained with UFH or LMWH in plasma can be used without any difference. The heparin calibrations proposed by the various manufacturers differ significantly, and this can have an important impact on exactness and accuracy of measured heparin concentrations. Reagents C and D use a calibration curve prepared with only LMWH spiked in a platelet depleted plasma pool. However, LMWH used by both manufacturers C and D are not the same as demonstrated by the different anti-FXa/Anti-FIIa ratios. LMWH is like certoparin for reagent C and like enoxaparin for reagent D. Conversely, reagents A and B propose a hybrid calibration curve obtained by mixing or combining UFH and LMWH in plasma. The anti-FXa/anti-FIIa ratios suggest that calibrators used for reagent B are obtained using a mixture of UFH and LMWH, the UFH concentration being predominant as shown by the low ratio obtained. In contrast, reagent A uses an alternate combination of plasmas containing UFH (calibrators 2 and 4) or LMWH (calibrators 3 and 5), with an anti-FXa/anti-FIIa ratio like that of certoparin. If UFH and LMWH calibration curves are not strictly superimposable, then the hybrid calibration curve is an intermediate curve which introduces some biases for both UFH

and LMWH samples. UFH concentrations tend then to be underestimated, as observed with reagents A and B, especially in the low range.

Lastly, the various anti-FXa reagents were used along with each manufacturer proposed instrument for reagents A, B and C, and have been used by adhering strictly to the instructions for use. Reagent D is proposed as a multiplatform reagent, with applications specifically developed for all the major instruments available. In this study, reagent D was combined with the Sysmex CS-5100 instrument.

When using homogenous (same manufacturer) reagent-instrument systems, reagent weaknesses can be masked by the assay software adaptation, or by introducing algorithms for optimizing the assay apparent performances. This approach is used for adjusting the intrinsic low anti-FXa activity present in all plasma samples, and which can be variable from plasma to plasma. In the absence of heparin, this intrinsic anti-FXa activity can account for 0 to 0.05 IU/ml in normal plasmas, and more rarely up to 0.10 IU. This background activity is likely due to the anti-FXa activity of TFPI, Protein S or the β -AT form. The anti-FXa heparin assay is an inverse relationship between absorbance change measured at 405 nM, and heparin concentration. Therefore, normal plasmas, whilst they are expected to have all the same basic absorbance in the absence of heparin, do not always show a zero anti-FXa activity. Assay systems can manage this variability by masking that effect and “starting to measure” the change in absorbance only from a threshold value, corresponding to plasmas with the highest anti-FXa intrinsic activity. The apparent heparin concentrations in all plasmas are then of 0 in the absence of heparin, but low concentrations of heparin, in the range 0 to 0.10 IU/mL, or even up to 0.15 IU/ml, can be missed, which contributes to the underestimation in the low range. This approach is of course not possible when the reagent is a multiplatform one, and no adjustment assay software can be used. Heparin concentrations measured in plasma are then obtained without any data treatment.

5. Conclusions

In this report we provide evidence on the usefulness of dextran sulfate for the anti-FXa assays used for measuring plasma concentrations of UFH or LMWH, as shown by the good correlation between all assays, designed with or without dextran sulfate, when plasma is obtained from CTAD collected blood, thus preventing from platelet activation and release of heparin neutralizing proteins, but not when blood is collected on citrate. Assay variability can also result from the heparin calibration type used, the exactness of UFH and LMWH superimposition of calibration curves and the assay software for treatment of assay raw data. Analyzing these factors can help for a better understanding of differences reported in many studies on heparin concentrations when measured with the various anti-FXa reagents.

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