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PHARMACOKINETIC PARAMETERS TO BE EVALUATED FOR SELECTED LOW MOLECULAR WEIGHT HEPARINs IN BIOEQUIVALENCE STUDIES

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ABBREVIATIONS AND ACRONYMS

LMWH: Low Molecular Weight Heparin
Heptest: Heparin Clotting Assay
APTT: Activated Partial Thrombin Time
TT: Thrombin Time
ACT: Activated Clotting Time
TFPI: Tissue Factor Pathway Inhibitor

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ABSTRACT

Bioequivalence needs to be established on healthy human volunteers for Low Molecular Weight Heparins (LMWHs) such as Dalteparin, Enoxaparin, Tinzaparin and Fondaparinux using Pharmacodynamic marker(s) for generic approval. Anti-Xa and anti-IIa activity are used to determine the activity of LMWHs (Dalteparin, Enoxaparin and Tinzaparin) and anti-Xa activity for Fondaparinux in biological samples for the assessment of its bioavailability. These are selected based on the pharmacodynamic activities of LMWHs. LMWHs exhibit their antithrombotic activity preferentially by inhibiting clotting Factor Xa, and to a lesser extent Factor IIa. On the other hand Fondaparinux is a synthetic and specific inhibitor of Factor-Xa and hence bioequivalence needs to be established for only anti-Xa activity. The pharmacodynamic data of anti-IIa activity need to be submitted for regulatory agency as supportive data of comparable therapeutic outcome for all LMWHs except Fondaparinux. In addition to the above, pharmacokinetic data of Heptest (Heparin clotting assay) and activated Partial Thromboplastin Time (aPTT) may also serve as a supportive evidence for establishing bioequivalence of LMWH formulations as there were no clear recommendations available.

INTRODUCTION: Low molecular weight heparins (LMWHs) are antithrombotic drugs obtained from unfractionated heparin by chemical or enzymatic hydrolysis [1]. They contain molecules with high and low affinity for antithrombin III [2]. These LMWHs inhibit blood coagulation by binding to antithrombin III, and the resulting complex inhibits clotting Factor Xa to a greater extent and Factor IIa (thrombin) to a lesser extent [3] (**Figure 1**).

Thus, when these are administered to human, they preferentially potentiate the inhibition of Factor Xa, while they barely affect the activated partial thromboplastin time (aPTT) [4].

LMWHs offers the advantage over heparin in having greater bioavailability (approximately 90%) [5], longer half-life (~ 4 to 6 hours), slower renal clearance, resistance to inactivation by platelet factor 4 [6] and relatively less number of serious adverse reactions particularly thrombocytopenia and thrombosis [7].

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The primary advantage of greater bioavailability of LMWHs over unfractionated heparins gives the provision for subcutaneous administration without the need for routine laboratory monitoring or dosage adjustment as with the case of heparin [8-10].

From the time of its introduction, in the early 1980s, they evolved as first line drugs and have begun to replace heparin in the treatment and prophylaxis of deep vein thrombosis and management of unstable coronary syndromes [11-17]. Most commonly used LMWHs are Dalteparin, Enoxaparin and Tinzaparin. These differ in their anti Xa: anti IIa ratio [Dalteparin (2.7:1), Enoxaparin (3.8:1) and Tinzaparin (1.9:1)] [18]. At higher doses these drugs are used to treat active thrombotic disease and at lower dose to prevent thrombosis. These are administered subcutaneously based on individual's body weight [19]. Now-a-days, Fondaparinux, a synthetic and specific anti-Xa inhibitor proved to be safe and efficacious in children with thromboembolic complications [20]. It is available as a sterile solution for subcutaneous administration [21].

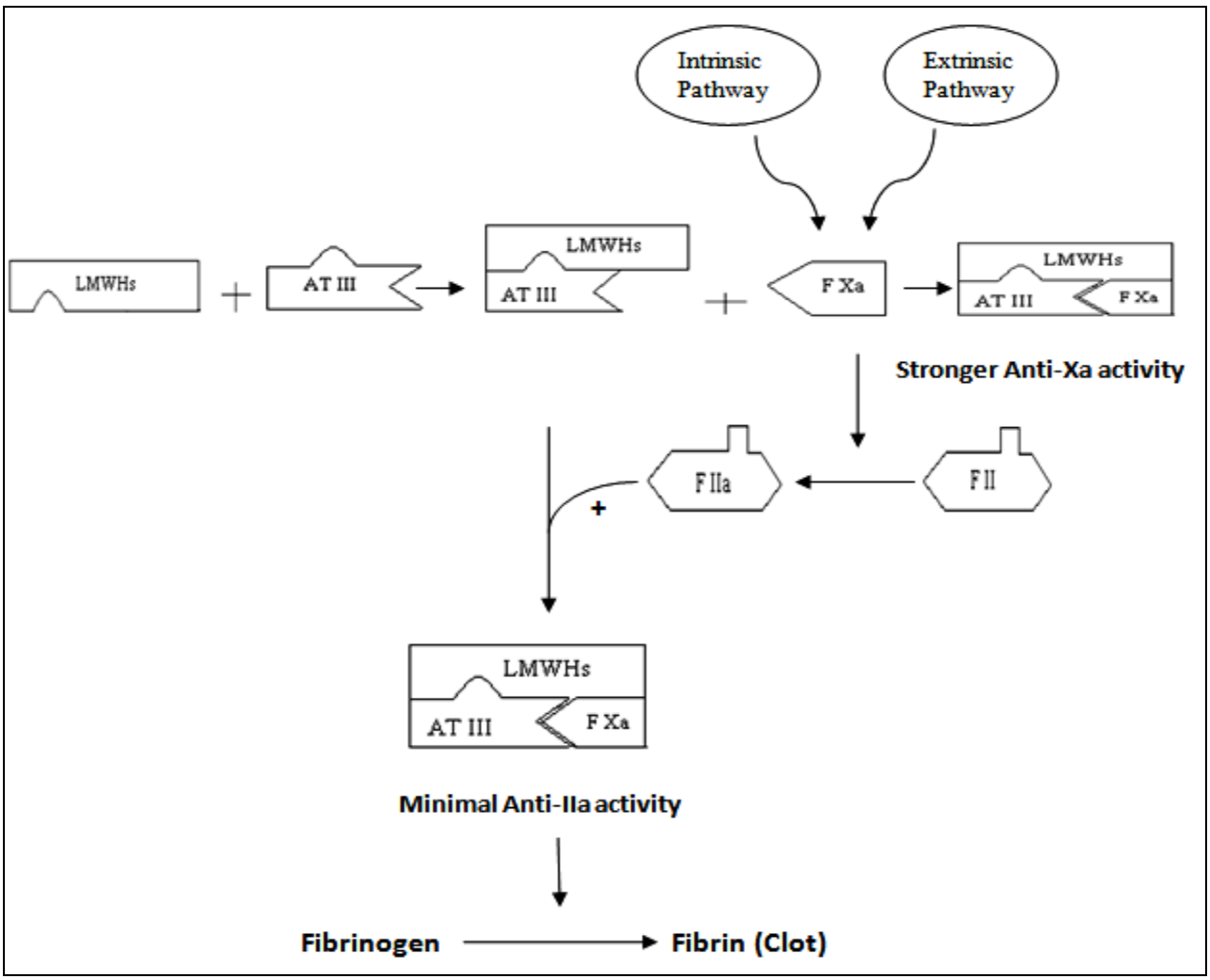

FIGURE 1: PHARMACODYNAMIC ACTIVITY OF LMWHs

Bioequivalence needs to be proved for a generic drug to be approved by the regulatory agency. A generic drug product is said to be bioequivalent to the Reference drug product if there is absence of statistically significant difference in the rate and extent of absorption of the active ingredient that is available at the site of drug action when administered at the same molar dose under similar experimental conditions [22]. In order to market a generic product of LMWHs, such comparative bioavailability studies need to be established [23]. Very little literature is available on the bioequivalence studies on LMWHs (Dalteparin, Enoxaparin, Tinzaparin and Fondaparinux). Hence an attempt was made to provide an insight into various aspects (such as study population, study restrictions and measurable pharmacodynamic (PD) markers) required for the conduct of bioequivalence studies for generic filing which might be of much interest for generic manufacturers working on LMWHs.

This review focus on the various pharmacodynamic parameters to be derived for proving bioequivalence of two different formulations of most commonly used LMWHs namely dalteparin, enoxaparin, tinzaparin and fondaparinux. A schematic representation of the way-forward to conduct a bioequivalence study of LMWHs is presented in **Figure 2**.

Study Population: Available literature data on LMWHs indicates that few studies have earlier been conducted on healthy volunteers. One such trial conducted in healthy Chinese male subjects where the study design includes an open-label, single-dose, randomized, two-period, two-sequence, crossover study under fasting conditions wherein along with planned bioequivalence, anti-Xa and anti-IIa activities, heparin clotting assay (Heptest) and activated partial thrombin time (aPTT) were also performed to check the sameness of the two formulations [24].

Reproduction studies with Dalteparin sodium at intravenous doses up to 2400 IU/kg (14,160 IU/m^2) in pregnant rats and 4800 IU/kg (40,800 IU/m^2) in pregnant rabbits did not produce any evidence of impaired fertility or harm to the fetuses. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed [25]. Therefore, the study population in bioequivalence study of LMWHs should include healthy adult male and non-pregnant, non-lactating female volunteers which are in accordance with the Food and Drug Administration (FDA) recommendation which states that "If the drug product is intended for use in both sexes, the sponsor should attempt to include similar proportions of males and females in the study" [26].

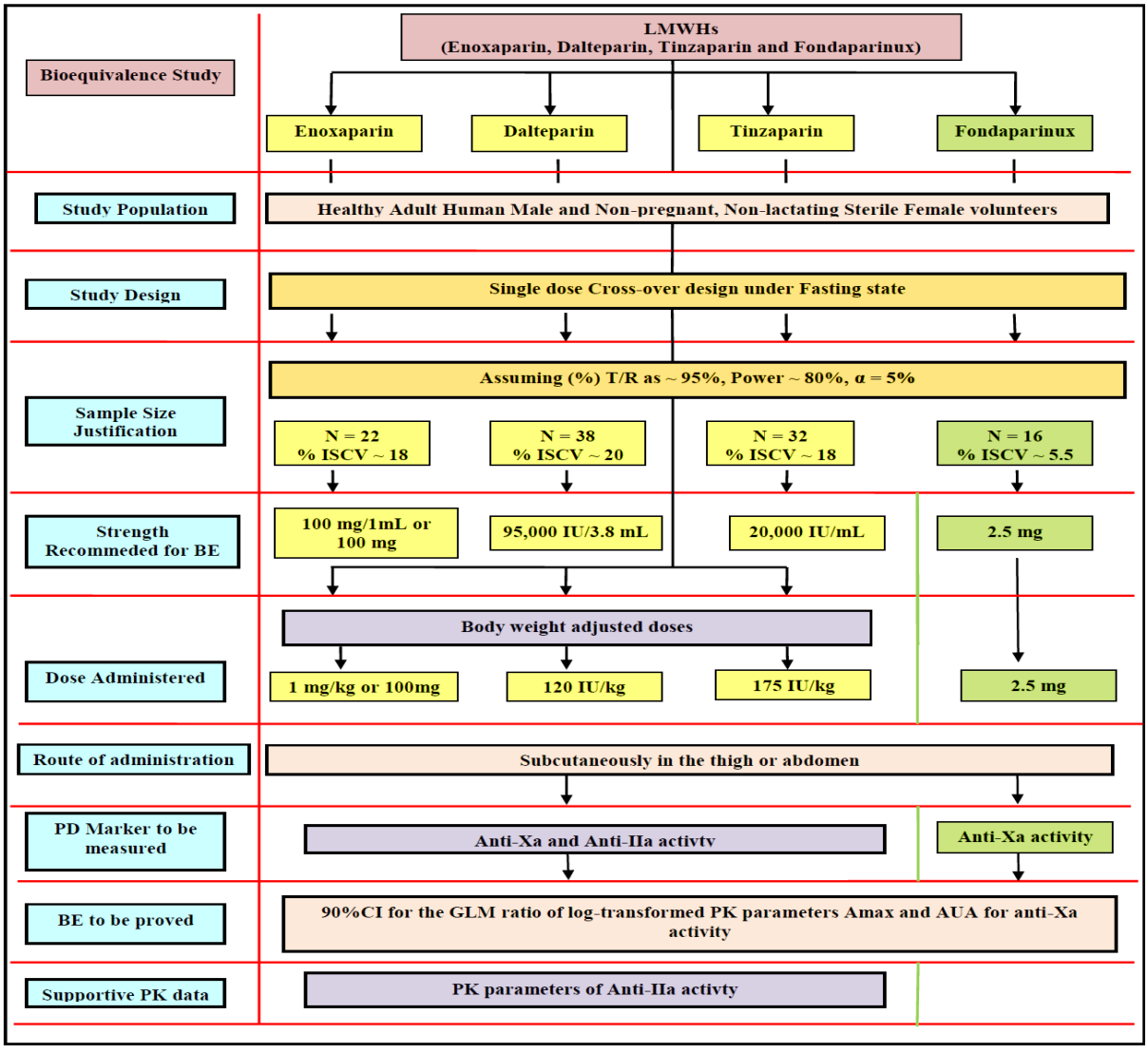

FIGURE 2: STUDY DESIGN AND CONDUCT OF BIOEQUIVALENCE STUDY OF LMWHs

Study Design: In comparative bioavailability studies, cross over remains the preferred design in order to minimize subject-by-subject variations and to minimize variability between drug treatments. Also single dose pharmacokinetic study is generally the most sensitive to changes in formulation related factors. Hence for studying bioequivalence of LMWHs (dalteparin, enoxaparin, tinzaparin and fondaparinux), the appropriate study design when two formulations are to be tested will be a randomized, single dose, crossover bioequivalence study under fasting conditions with pharmacodynamic end point(s). Sufficient number of healthy adult human volunteers who are willing to provide written informed consent and comply with the study restrictions need to be enrolled.

Dose Selection: The dose provided below is for generic developed for marketing in U.S. The dose to be tested depend on subject safety and may also vary according to the country for which generic will be filed. In general, the highest marketed strength need to be subjected for BE testing.

1. **Dalteparin [25]:** Doses of dalteparin sodium injection up to 10,000 anti-Factor Xa IU administered subcutaneously as a single dose or two 5000 IU doses 12 hrs apart to healthy subjects do not produce a significant change in platelet aggregation, fibrinolysis, or global clotting tests such as prothrombin time (PT), thrombin time (TT) or aPTT. Hence for comparative bioavailability evaluations, the dose provided in FDA (Food and Drug Administration) label (a bolus dose of 120 IU/kg or 10000 IU from the label potency (95000IU/3.8 mL)) need to be selected for bioequivalence testing. Dosage should not exceed 10,000 IU in any of the subjects.

2. **Enoxaparin [27]:** A dose of 100 mg of Enoxaparin provided by the OGD (Office of Generic Drugs) is found to be independent of variations in body weight in various studies. Also a bolus dose of 1 mg/Kg body weight or 100 mg dose can be chosen for bioequivalence testing. Dosage should not exceed 100 mg in any of the subjects.

3. **Tinzaparin [28]**: The Reference Listed Drug (RLD) strength in U.S is 20,000 IU anti-Xa/mL. The recommended dose for deep vein thrombosis (DVT) is 175 IU/kg body weight and this dose can be chosen for bioequivalence studies as this is proved to be safe in healthy volunteers.

4. **Fondaparinux [29]**: A dose of 2.5 mg subcutaneously is recommended for prophylaxis of deep vein thrombosis and this can be used in bioequivalence studies as it is proved to be safe.

Drug Administration: Subjects need to fast overnight prior to administration of study medication in each period. The dose to be administered is calculated as per the subject's body weight (except for fondaparinux). A single dose [120 IU/kg (Dalteparin sodium); 1 mg/kg (Enoxaparin sodium); 175 IU/kg (Tinzaparin sodium) and 2.5 mg (Fondaparinux)] of either test or reference product is administered subcutaneously to each subject as per the randomization schedule in each period. Subject receives an identical dose in both the periods.

Prior to administration, the drug products need to be inspected visually for particulate matter and discoloration. The subcutaneous injection should be injected in a U-shaped area around the navel or the upper outer side of the thigh or the upper outer quadrangle of the buttock. The dosing need to be done by trained study personnel. The entire length of the needle should be inserted at a 45 to 90 degree angle. The injected dose needs to be administered at the same site but not at the same side in the second period. [For example: if the injection is given in 12 O'clock position of the navel in first period, then in second period it should be given at 6 O'clock position below the navel]. A washout period of atleast 7 days between two consecutive dosing periods is sufficient to prevent drug carry over.

Study Restrictions: In order to standardize the study conditions, the following restrictions may be maintained during the study periods. Subjects must be instructed to abstain from consuming any alcoholic products, xanthine-containing food and/or beverages (like chocolate, tea, coffee, cola drinks), grapefruit juice, smoking, chewing tobacco, tobacco mixed masala, pan masala, gutkha, supari (betel nut) for a predetermined period (say for example at least 24 hours) prior to drug administration till last sample collection in each period.

Drinking water may be restricted from 1 hr predose until 1-hr post dose, and sitting posture for at least first two hrs following drug administration in each period.

Safety Assessment: Subject safety needs to be ensured at each stage of the study duration. Safety can be assessed through measurement of vitals (blood pressure, pulse rate), verifying subject's well-being, and/or performing physical or clinical examination at various stages of the study conduct and as when the physician feels it necessary. In addition to the above mentioned safety steps, the study may necessitates certain laboratory tests namely aPTT, TT (thrombin time) and INR (international normalized ratio) at the time of screening and ACT (Activated Clotting Time) estimation at the time of check-in and at regular intervals following drug administration in each period to ensure the safety of the subjects which indeed will be as per the physician discretion.

ACT is a bedside assay most often used in emergency situations [26] to provide rough estimation of blood level of LMWH whereas aPTT is a lab-based test which is a good predictor of LMWH concentration. Although the laboratory-based aPTT has a stronger correlation to heparin concentration than the bedside-based ACT, both (aPTT and ACT) are used to monitor anticoagulant activities.

Urine scan for drugs of abuse and breath test for alcohol consumption need to be done prior to check-in of each period. For female subjects, urine or serum pregnancy test is to be done at the check-in of each period and checkout of last period.

Blood Sampling Schedule: Due to difficulties in measuring LMWH concentrations directly, conventional pharmacokinetic studies cannot be performed. Instead, the absorption and elimination of LMWHs are studied by using Pharmacodynamic tests which includes measurement of anti-FXa and anti-FIIa activities. LMWHs potentiate preferentially the inhibition of coagulation Factor Xa, with only a slight inhibition of Factor IIa. Hence to determine the pharmacokinetic parameters accurately for pharmacodynamic markers of LMWH, blood samples need to be collected for each subject at appropriate intervals in each period.

Appropriate sampling schedule to adequately characterize all the four pharmacodynamic markers is as follows:

1. **For Dalteparin [25]**: The venous blood samples may be withdrawn at pre-dose (0.0) and at 0.5, 1, 2, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8.0, 10, 12, 14, 16, 24 and 36 hrs following drug administration in each period. It is observed that the 36 hrs postdose sample collection point may contribute to more variability based on our previous experience.

2. **For Enoxaparin [24]**: Predose (0.0) and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16 and 24 hrs postdose.

3. **For Tinzaparin [28]**: Predose (0.0), 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24 and 30 hrs post dose.

4. **For Fondaparinux [30]**: Predose (0.0), 0.25, 0.5, 0.75, 1, 1.33, 1.67, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, 36, 48, 60 and 72 hrs post dose.

Bioanalytical Procedure: Pharmacodynamic markers namely anti-Xa and anti-IIa activities will be used to determine the activity of LMWH (Dalteparin, Enoxaparin and tinzaparin) in biological samples using validated bioassays using chromogenic methodologies for anti-Xa and anti-IIa. Apart from these two primary tests, clot based assay for Heptest using Heparin clotting assay and aPTT also were determined in earlier studies prior to availability of guidance on Enoxaparin sodium injection [25, 32-33]. These are based on the Pharmacodynamic activities of LMWHs.

1. **Anti-Xa and Anti-IIa Assays:** These are termed as antiprotease assays. Due to the high anti-Xa activity of LMWH, anti-Xa assays are commonly used to monitor LMWH levels, [32] but this may not accurately reflect the anticoagulant action because LMWHs also inhibit Factor IIa. Hence anti-IIa assay also need to be carried out.

2. **Heptest:** The Heptest is a clotting assay that is sensitive to anti-Xa and anti-IIa activity, as well as inhibition of the extrinsic pathway by LMWH-stimulated release of tissue factor pathway inhibitor (TFPI) [34]. LMWH is a mixture of various oligosaccharides endowed with various anti-Xa and anti-IIa properties. Hence apart from measuring anti-Xa activity, as LMWH also inhibits

Factor IIa, accurate tests of anticoagulant actions must be able to measure anti- IIa activity in addition to anti-Xa activity.

a. **Release of tissue factor pathway inhibitor (TFPI):** The LMWHs stimulate the release of TFPI from the endothelium and enhance its inhibitory activity against Factor Xa. The activity of Heptest is not only as a result of anti-Xa and anti-IIa activities but also from the ability of LMWHs to induce TFPI release from endothelium. *In-vitro* studies have demonstrated that at high concentrations (2–5 mg/mL) of LMWH fractions, the Heptest can measure both the anti-Xa and the anti-IIa activities of the compounds, whereas at the lower concentrations (2 mg/mL) this test reflects predominantly the anti-Xa activity. Hence Anti-IIa assays need to be separately carried out.

3. **Anti-IIa Assays:** Plasma thrombin neutralization assay: It has also been used to measure LMWH and to detect low concentrations to which chromogenic assays are insensitive.

a. Monitoring of the anticoagulant Effect [35]: Unlike standard heparin, LMWHs have little effect on the activated partial thromboplastin time (aPPT) or celite-activated clotting time (ACT). Results of any such tests after the administration of LMWHs should therefore be interpreted with caution.

4. **Activated partial thromboplastin time** (**aPTT)**: The aPTT is a global clotting assay, which is used to measure inhibition of coagulation factors in the intrinsic pathway and is commonly used to monitor heparin therapy.

Pharmacodynamic (PD) parameters and their calculation procedure: The primary and secondary pharmacodynamic (PD) parameters to be determined for the LMWHs (Dalteparin, Enoxaparin and Tinzaparin) are summarized in **Table 1** and for Fondaparinux in **Table 2**. The Pharmacokinetic parameters for the pharmacodynamic markers can be computed by any pharmacokinetic software such as Win-Nonlin Professional Software (Pharsight Corporation, USA) using non-compartmental model. As Heptest (an indicator of relative amount of anti-Xa & anti-IIa activity) & aPTT (a safety indicator) are also major contributors of anticoagulant activities of LMWHs, the pharmacokinetic parameters of Heptest (A_{max}, AUA_{0-t} and $AUA0_{0-\infty}$) and aPTT ($(\Delta t)_{max}$, $AU(\Delta t)$ can be provided as a supportive evidence for the regulatory agency for proving BE of LMWHs. Hence their calculation procedure is briefed in this review.

TABLE 1: PHARMACOKINETIC PARAMETERS FOR THE PHARMACODYNAMIC MARKERS IN BIOEQUIVALENCE STUDIES OF LMWHS (DALTEPARIN, ENOXAPARIN AND TINZAPARIN)

PD Marker	Nature of PD Parameter	PD Parameter
Anti-FXa and Anti-FIIa, Heptest*	Primary Parameter	A_{max}, AUA_{0-t} and $AUA_{0-\infty}$
	Secondary Parameters	K_{el}, $t_{1/2}$, t_{max}

"*" PK data of Heptest is not a mandatory requirement for proving BE of LMWHs.

TABLE 2: PHARMACOKINETIC PARAMETERS FOR THE PHARMACODYNAMIC MARKERS IN FONDAPARINUX BIOEQUIVALENCE STUDIES

Pharmacodynamic (PD) Marker	Nature of PD Parameter	PD Parameter
Anti-FXa	Primary Parameter	A_{max}, AUA_{0-t} and $AUA_{0-\infty}$
	Secondary Parameters	K_{el}, $t_{1/2}$,, t_{max}

PK Parameters for Anti-FXa, Anti-FIIa and Heptest [24]:

A_{max} and T_{max}: The maximum measured plasma activity A_{max}, for the activity time profile will be determined observationally as the peak activity for each subject in each treatment. The time of maximum activity, T_{max}, will be determined as the time corresponding to A_{max}.

AUA_{0-t} and $AUA_{0-\infty}$: AUA_{0-t} is the area under the plasma activity versus time curve, from time zero to the last measurable time point and $AUA_{0-\infty}$ is area under the plasma activity versus time curve, from time zero to infinity. These are calculated using the linear trapezoidal method.

Half-life ($t_{1/2}$): Elimination or terminal half-life ($t_{1/2}$) will be calculated using the following formula:

$$t_{1/2} = \frac{0.693}{K_{el}}$$

Where (K_{el}) is the first order elimination or terminal rate constant, the value of which can be determined by a non-compartmental analysis using WinNonlin.

PK Parameters for For aPTT: Pharmacokinetic (PK) parameters: $(\Delta t)_{max}$, AU(Δt) and t_{max} were to be calculated.

$(\Delta t)_{max}$: It is the maximum measured change in clotting time compared to baseline. Hence baseline value is measured to determine $(\Delta t)_{max.}$

AU(Δt): AU(Δt) is the area under the curve for a plot of change in clotting time from baseline versus time.

Calculation Procedure For $(\Delta t)_{max}$, AU(Δt) for aPTT: Baseline aPTT value will be the sample collected at pre-dose i.e., 0.00 hr. aPTT is used to test the amount of time taken by blood to clot. LMWHs prolongs the time required for the blood sample to clot. i.e., it prolongs the clotting time. Therefore, upon drug administration, all the blood samples taken at different time points may show an increase in the clotting time as compared to baseline value. aPTT is to be measured at baseline as well as at serial time points after drug administration. Baseline aPTT value will be subtracted from aPTT obtained at various time points and the difference will be presented as Δt values. If Δt value found to be negative, then it will be set to zero. A plot of time (t) versus Δt will be plotted. Area under the curve for a plot of change in clotting time from baseline versus time will be AU(Δt).

Bioequivalence Criteria for LMWHs: Based on the statistical analysis, the test product will be concluded bioequivalent to the reference product if the 90% confidence interval for the ratio of the geometric least squares means of log-transformed pharmacokinetic parameters A_{max}, $AUA_{0\text{-}t}$ and $AUA_{0\text{-}\infty}$ for anti-Xa fall within the regulatory set BE criteria, say for ex. 80.00–125.00% for generic filing in U.S [36], A_{max} and $AUA_{0\text{-}t}$ in European Union (EU) [37] and Canada [38] respectively.

Tinzaparin: Till date, there were no clear-cut recommendations provided by any regulatory agency on the bioequivalence criteria to be submitted for generic LMWHs (except enoxaparin and dalteparin). The BE criteria can be based on the available FDA (Food and Drug Administration) review data and OGD (Office of Generic Drugs) guidance available for enoxaparin and and dalteparin [39] where BE is based primarily on anti-Xa activity. The pharmacokinetic data of anti-IIa activity need to be submitted as supportive data. Also the point estimate (test/reference) of ln transformed $AUA_{0\text{-}inf}$ (anti-Xa) / $AUA_{0\text{-}inf}$ (anti-IIa) ratio may be submitted to provide complete data.

CONCLUSION(S): Bioequivalence studies for LMWHs (Dalteparin, Enoxaparin, Tinzaparin and Fondaparinux) are to be conducted on healthy, adult, human subjects under fasting conditions using pharmacodynamic end point(s). For LMWHs, the test formulation will be assessed bioequivalent to the reference formulation if the 90% Confidence Interval for the geometric least squares mean ratio of log-transformed pharmacokinetic parameters (A_{max}, $AUA_{0\text{-}t}$ and $AUA_{0\text{-}inf}$) for anti-Xa of test and reference formulations meet the regulatory set criteria. The pharmacokinetic data of anti-IIa activity (for Enoxaparin, Dalteparin and Tinzaparin) need to be submitted as supportive evidence of comparable therapeutic outcome.

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