Validation of Methods for the Determination of Low Molecular Heparin Anti-IIa Activity Assays for Analysis of Enoxaparin

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Introduction

The activity of Enoxaparin is dependent upon its binding to anti-thrombin (AT). Binding induces a conformational change in the molecule which accelerates its inhibitory activity. As LMWH are heterogeneous molecules, it is not possible to quantify them directly; therefore, we report here on the development and validation of chromogenic activity assays for the determination of Enoxaparin using anti-IIa activity in human plasma.

Materials and methods

The Anti-IIa activity assay is carried out by addition of standards, quality controls and samples to a human anti thrombin III reagent (25µL; 375µL). After mixing, the mixture is allowed to incubate at 37˚ C for 15 minutes. Then an aliquote removed and additional (200µL) of human anti thrombin III reagent is added and allowed to incubate for 2 minutes. Bovine thrombin is added (200 µL) and allowed to incubate for 2 minutes. Spectrozyme TH substrate (200 µL) is then added and allowed to incubate for 2 minutes. Finally, glacial acid (200 µL) is added to stop the reaction. The plate is then read at 405 and 450 nm

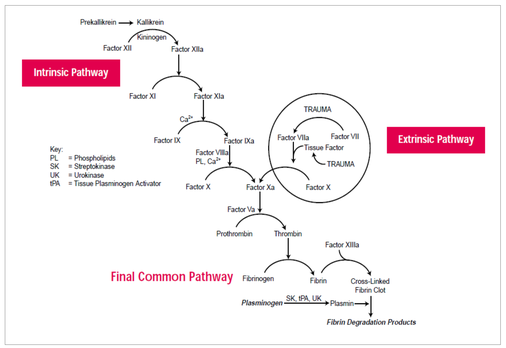

Figure 1. Hemostasis process in humans
Source: Majerus, P.W., et al. 1990. In The Pharmacological Basis of Therapeutics, 8th Edition (Gilman, A.G., et al.) pp. 1311-1331, New York, Pergamon Press.

Results

For the anti-IIa method the calibration range was from 0.0400 to 0.500 IU/mL with a LLOQ of 0.0400 IU/mL. The accuracy ranged from -8.6 to 3.6% and an intra-precision, ranged from 1.9 to 21.3%, an inter-precision ranged from -8.6 to 21.3% and a total error ranged from 4.4 to 22.6%. Dilutional linearity was acceptable for up to 10-fold dilution and no hook effect was observed. Enoxaparin LMWH using anti-IIa is stable in human plasma samples held in polypropylene tubes for 25.5 hours and 24.5 hours at ambient temperature (~25˚ C) and at approximately 4˚ C, respectively. Enoxaparin using anti-IIa stablity was proven in human plasma stored at approximately 80˚ C in polypropylene tubes for up to 57 days and for up to 4 freeze/thaw cycles.

		Nominal Concentration (ng/mL)				
		QC1	QC2	QC3	QC4	QC5
Characteristic	Statistic	0.0400	0.100	0.200	0.400	0.500
# Results	N	41	41	40	42	42
Accuracy	Mean Bias (%RE)	0.2	1.4	3.6	1.9	-8.6
Precision	Intrabatch (%CV)	21.3	2.8	1.9	2.1	2.5
Total Error	|Mean| + Interbatch	22.582	4.200	5.504	4.932	12.890

Table 1. Accuracy and precision Summary
Total Error for Precision and Accuracy from LBA Spreadsheet for Enoxaparin Low Molecular Weight Heparin using Anti-IIa in Human Plasma

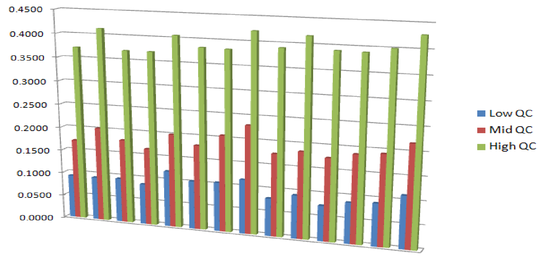

Figure 2. Analytical Performance of Quality Control Samples for the Determination of Enoxaparin Low Molecular Weight Heparin using Anti-IIa in Human Plasma

All values within acceptance criteria: ±20.0% RE for all QC samples

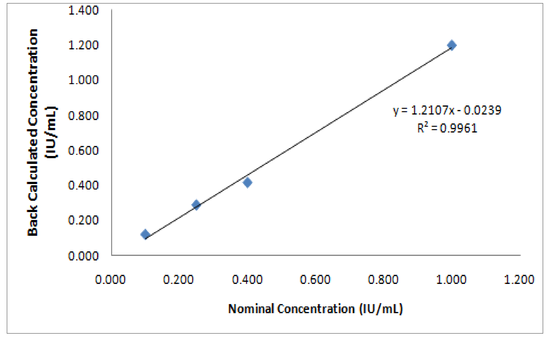

Figure 3. Linearity of Dilution and Hook effect for Enoxaparin Low Molecular Weight Heparin using Anti-IIa in Human Plasma

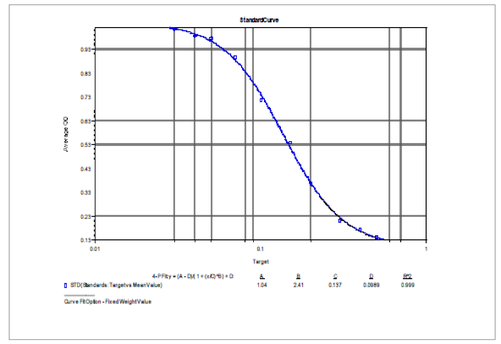

Figure 4. Representative Standard Curve of Enoxaparin Low Molecular Weight Heparin using Anti-IIa in Human Plasma

Stability Experiment	Low Concentration (0.1 IU/mL)		High Concentration (0.4 IU/mL)	
	RE (%)	CV (%)	RE (%)	CV (%)
Short Term Stability at room temperature (25.5 hours)	-10	3.99	2.5	1.97
Short Term Stability at 4°C (24.5 hours)	-8.9	2.84	0.75	2.44
Freeze thaw Stability at -20°C (4 cycles)	-9.6	9.99	1.75	1.11
Freeze thaw Stability at -80°C (4 cycles)	-4.8	2.7	-1.75	1.55
Long term Stability at -80°C (25 days)	1	5.09	7	0.404

Table 2. Stability of Enoxaparin Low Molecular Weight Heparin using Anti-IIa in Human Plasma

Conclusions

The validated Chromogenic assays for the determination of Enoxaparin using anti-IIa activity in human plasma successfully met all standard assay-validation parameters and were suitable for use in bioequivalence studies.

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