Singlicate well assay for the analysis of Enoxaparin Pharmacodynamic Biomarkers (Anti-Factor Xa, Anti-Factor IIa, and TFPI) for the Bioequivalence study.

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INTRODUCTION

Enoxaparin is a low molecular weight heparin (LMWH) used to treat and prevent venous thrombosis. LMWH acts as an anticoagulant by inactivating factor Xa and factor IIa and promoting the release of tissue factor pathway inhibitor (TFPI), preventing blood coagulation. Enoxaparin is a complex mixture of oligosaccharides with variations in chemical structure and size, making direct conventional pharmacokinetic measurement unfeasible. Instead, regulatory requirements accept measuring pharmacodynamic markers such as anti-factor Xa, anti-factor IIa, and TFPI for comparison between the biosimilar and the reference LMWH.

We have developed the single well assay, and microtiter plates were read using Softmax Pro GXP software. All three methods are optimized to improve assay performance, reproducibility, and high throughput using the single well.

CHALLENGE

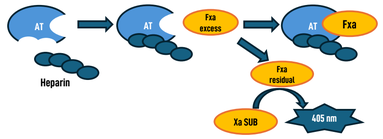
The challenge lies in meeting regulatory expectations, particularly regarding the analytical validation criteria for biomarker assays. While pharmacokinetic (PK) assays and in vitro diagnostic devices (IVD) have well-defined acceptance criteria, these standards may not seamlessly apply to biomarker assays due to their unique requirements. Biomarker assays demand distinct expectations, necessitating a refinement of analytical validation to suit the specific context of drug development.

NOVEL ASPECTS

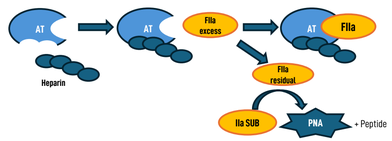
- Methods utilize the principles of regulated pharmacokinetics (PK) and ligand binding assay (LBA) design to repurpose diagnostic instrument commercial kit assays. This involves incorporating features such as a minimum of six-point calibration curves (CC), multiple layers of quality controls (QCs), and comprehensive validation parameters including accuracy, precision, selectivity, specificity, and stability.
- Implements singlicate analysis by utilizing single wells instead of duplicate wells, streamlining the assay process for efficiency and resource optimization.
- Incorporates ISR for assessing reproducibility, a practice not typically performed for biomarkers. This ensures robustness and reliability by evaluating the consistency of results across independent sample analyses.

METHODS

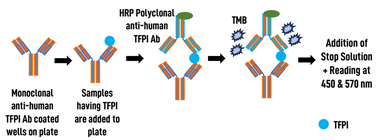
Anti-Factor Xa assay



Anti-Factor IIa assay



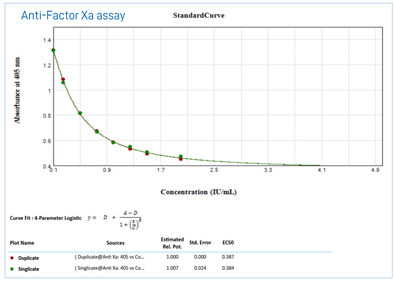
TFPI assay



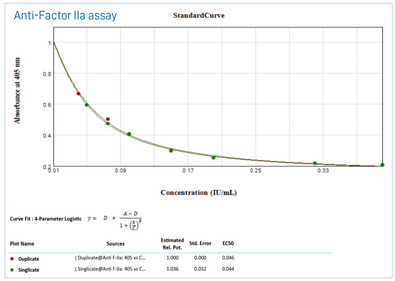
RESULTS

Comparison of Calibration curve, Duplicate vs Singlicate

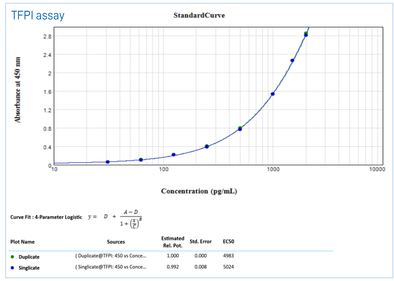
Anti-Factor Xa assay



Anti-Factor IIa assay



TFPI assay



Comparison of Accuracy and Precision, Duplicate vs Singlicate

Anti-factor Xa

Inter assay (n=24)

	Duplicate		Singlicate	
QC ID	Precision (%CV)	Accuracy (%RE)	Precision (%CV)	Accuracy (%RE)
LLOQQC	9.9	1.0	7.9	0.5
LQC	7.0	-9.4	3.9	1.2
MQC	5.6	-6.2	2.9	3.2
HQC	3.5	1.2	4.3	0.9
ULOQQC	7.4	7.1	4.1	0.8

Anti-factor IIa

Inter assay (n=24)

	Duplicate		Singlicate	
QC ID	Precision (%CV)	Accuracy (%RE)	Precision (%CV)	Accuracy (%RE)
LLOQQC	10.3	-2.8	10.1	0.8
LQC	5.4	3.0	4.6	-4.7
MQC	6.0	3.5	3.9	-2.7
HQC	5.3	3.6	3.5	-1.1
ULOQQC	8.1	0.0	4.5	-0.9

TFPI

Inter assay (n=24)

	Duplicate		Singlicate	
QC ID	Precision (%CV)	Accuracy (%RE)	Precision (%CV)	Accuracy (%RE)
HQC	2.0	1.5	4.8	1.5
MQC	1.7	1.6	5.9	1.3
LQC	3.6	2.7	9.1	2.1
Matrix QC-1	10.7	3.5	9.7	3.4
Matrix QC-2	8.3	-1.3	7.6	6.2

Accuracy and Precision, Method Validation Singlicate analysis

Anti-factor Xa

Intra assay

QC ID	Nominal (IU/mL)	Average Conc. (IU/mL)	SD	%CV	%RE	%TE
LLOQQC	0.100	0.107	0.005	4.7	7.0	11.7
LQC	0.300	0.312	0.012	3.9	3.8	7.7
MQC	0.700	0.738	0.011	1.5	5.5	7.0
HQC	1.800	1.823	0.039	2.1	1.3	3.4
ULOQQC	2.400	2.462	0.029	1.2	2.6	3.8

Inter assay (n=24)

QC ID	Nominal (IU/mL)	Average Conc. (IU/mL)	SD	%CV	%RE	%TE
LLOQQC	0.100	0.101	0.008	7.9	0.5	8.4
LQC	0.300	0.304	0.012	3.9	1.2	5.1
MQC	0.700	0.722	0.021	2.9	3.2	6.1
HQC	1.800	1.816	0.079	4.3	0.9	5.2
ULOQQC	2.400	2.419	0.098	4.1	0.8	4.9

Anti-factor IIa

Intra assay

QC ID	Nominal (IU/mL)	Average Conc. (IU/mL)	SD	%CV	%RE	%TE
LLOQQC	0.040	0.040	0.001	3.3	-1.3	4.6
LQC	0.120	0.112	0.002	1.6	-6.7	8.3
MQC	0.210	0.195	0.002	0.9	-7.4	8.3
HQC	0.300	0.286	0.003	1.1	-4.8	5.9
ULOQQC	0.400	0.390	0.002	0.4	-2.4	2.8

Inter assay (n=24)

QC ID	Nominal (IU/mL)	Average Conc. (IU/mL)	SD	%CV	%RE	%TE
LLOQQC	0.040	0.040	0.004	10.1	0.8	10.9
LQC	0.120	0.114	0.005	4.6	-4.7	9.3
MQC	0.210	0.205	0.008	3.9	-2.7	6.3
HQC	0.300	0.297	0.010	3.5	-1.1	4.6
ULOQQC	0.400	0.396	0.018	4.5	-0.9	5.4

TFPI

Intra assay

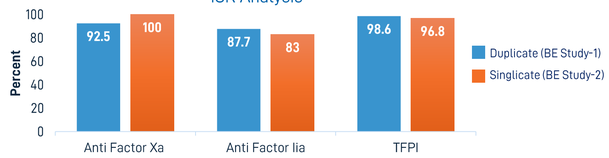
QC ID	Nominal (pg/mL)	Average Conc. (pg/mL)	SD	%CV	%RE	%TE
LLOQ QC	62.500	59.654	2.147	3.6	-4.4	8.2
LQC	180.000	185.523	3.570	1.9	3.1	5.0
MQC	440.000	438.373	8.555	2.0	-0.4	2.4
HQC	1400.000	1372.738	26.377	1.9	-1.9	3.8
ULOQ QC	2000.000	1956.333	46.745	2.4	-2.2	4.6
Matrix QC-1	23501.819	23832.957	418.792	1.8	1.4	3.2
Matrix QC-2	57536.932	62777.184	1788.585	2.8	9.1	11.9

Inter assay (n=24)

QC ID	Nominal (pg/mL)	Average Conc. (pg/mL)	SD	%CV	%RE	%TE
LLOQ QC	62.500	59.765	8.094	13.5	-4.4	17.9
LQC	180.000	183.714	16.771	9.1	2.1	11.2
MQC	440.000	445.832	26.319	5.9	1.3	7.2
HQC	1400.000	1420.847	68.111	4.8	1.5	6.3
ULOQ QC	2000.000	1972.013	81.158	4.1	-1.4	5.5
Matrix QC-1	23501.819	24294.812	2359.296	9.7	3.4	13.1
Matrix QC-2	57536.932	61119.659	4619.056	7.6	6.2	13.8

INCURRED SAMPLE REANALYSIS

ISR Analysis



CONCLUSIONS

All three Pharmacodynamic Biomarkers (Anti-Factor Xa, Anti-Factor IIa, and TFPI) methods were validated in Singlicate (single well measurement). Although all analytes are biomarkers, experimental design and acceptance criteria suggested for the Ligand Binding Assay (LBA) have been followed. Applicable validation parameters, i.e., precision and accuracy, sensitivity, matrix effect, hemolysis impact, stabilities in matrix, and dilution integrity, have been performed and meet the acceptance criteria suggested for the Ligand Binding Assay (LBA).

The method was successfully applied to one pivotal study without any challenge and assay failure rate is negligible, endorsing the robustness of the single well approach. Furthermore, assay reproducibility of in-vivo samples is proven using Incurred Sample Reproducibility experiment for anti-factor Xa, IIa, and TFPI and acceptance is found 100.0 %, 83.0 % and 96.8 % respectively.

The results of this clinical study conclusively showed that the Enoxaparin manufactured by the sponsor is highly similar to the innovator reference Enoxaparin in all primary (anti-FXa) and secondary (anti-FIIa and TFPI activity) PD parameters.

Validated methods in Singlicate demonstrate accuracy, precision, robustness, cost-effectiveness, and increased throughput. In summary, there is no meaningful difference between single well assay and duplicate well assay if the assay is robust enough to tolerate marginal analytical variation.

REFERENCES

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- Ye, Z., Tu, J., Midde, K., Edwards, M., & Bennett, P. (2018). Singlicate analysis: should this be the default for biomarker measurements using ligand-binding assays?. Bioanalysis, 10(12), 909–912.
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