Bioanalytical Challenges in developing and validating a biomarker assay for Tissue Factor Pathway inhibitor (TFPI) using commercial kit.

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Introduction

Human TFPI, also known as lipoprotein associated coagulation inhibitor (LACI) and extrinsic pathway inhibitor (EPI), is a physiological inhibitor of extrinsic pathway of coagulation and has biological functions of anticoagulation and anti inflammation. Tissue factor pathway inhibitor (TFPI) is a key regulator of the extrinsic coagulation pathway. It inhibits coagulation through the formation of a quaternary complex with factor X, tissue factor, and factor VII, preventing factor X from activation which in turn does not convert prothrombin to thrombin and hence no fibrin clot formation takes place.

The TFPI assay is a quantitative biomarker assay which makes use of a quantitative immunoassay. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TFPI has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TFPI present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human TFPI is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TFPI bound in the initial step. The color development is stopped and the intensity of the color is measured.

Challenges

There are some challenges distinctively related to quantitative biomarker assay validation were met at the start of method development and decision making. The major dilemma that were experienced at the beginning for a quantitative analysis are illustrated below.

Major Issue / Challenge	Description
Commercial Research based kit	• Procured research based kit from reputed vendor. No quality control available in the kit or purified protein to prepare QCs in bulk.
Reference Standard	• There was no innovator drug available to be used as reference standard. • Procured recombinant protein from reputed vendor.
Preparation of Quality Controls	• Quality Controls at two levels were prepared in human citrated plasma. Plasma samples were screened for high and low endogenous TFPI concentrations. High and low matrix (MC) that is plasma controls were used for precision, accuracy and analyte stability. • Three levels of TFPI quality control spanning the calibration range were procured and used in the assay.

QCs prepared using purified recombinant human TFPI

Sample	Mean OD	CV	% Error
High Control-1	1.128	0.9	-59.023
High Control-2	1.129	0.8	-58.976
Low Control-1	0.074	1.2	-56.763
Low Control-2	0.075	0.8	-55.915
Mid Control-1	0.529	0.9	-59.237
Mid Control-1	0.52	5.5	-59.935

Fit For Purpose Method Validation Approach:

A fit-for-purpose method validation approach that addresses all analytical considerations were used to conduct a study as a part of pharmcodynamic parameter for low molecular weight heparin (LMWH). Biomarkers can be used for a wide variety of purposes during drug development; therefore a fit-for-purpose approach should be used when evaluating the extent of method validation that is appropriate. Method validation for biomarker assays should address the same questions as method validation for PK assays. The approach used for PK assays should be the starting point for validation of biomarker assays, although regulatory realizes that some characteristics may not apply or that different considerations may need to be addressed.

- A research based commercial kit from reputed vendor was selected and used for validation.
- The kit prepares calibration standards in buffered protein base instead of plasma because of high endogenous level.
- Results obtained using natural human TFPI showed linear curves that were parallel to the Standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human TFPI.
- Five levels of quality control samples were prepared.
- Quality controls at two concentrations in plasma i.e. one high and one low were prepared namely high and low matrix (MC) control. These controls are high and low concentration of naturally occurring TFPI which have been accurately estimated in several assays.
- Three quality controls of high, medium and low TFPI concentration spanning the calibration range were procured and used. The actual concentration were accurately estimated in several assays.
- Precision and accuracy, stability, MRD, selectivity, assay range were validated.

TFPI Method Summary:

Analyte	:	Tissue Factor Pathway Inhibitor (TFPI)
ELISA Kit	:	Human TFPI Quantikine ELISA kit
Assay technique	:	Quantitative sandwich imunoassay
Minimum Required Dilution	:	100 fold
Calibration curve range	:	31.25 – 2000 pg/mL
Quality Controls (pg/mL)	:	31615.225 (High MC), 16857.065 (Low MC), 905.159 (HQC), 482.542 (MQC), 153.206 (LQC)
Lower limit of quantitation	:	31.25 pg/mL

Calibration Standards and curve

The calibration standards could not be spiked in human plasma for preparation of calibration curve because of very high endogenous TFPI level. Hence the standards for calibration curve were prepared in calibrator diluent provided in kit.

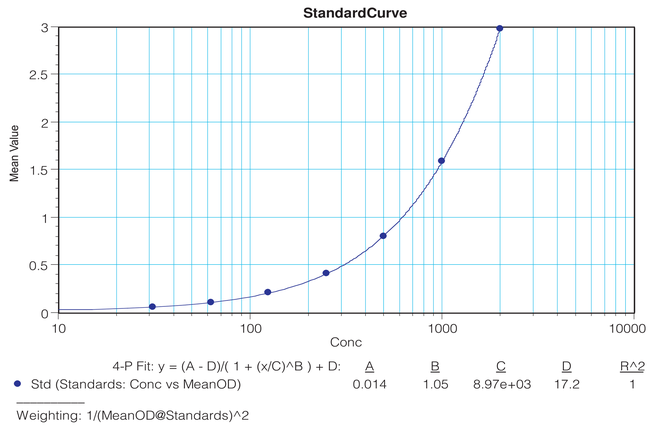

Fig 1: Representative 4-PL calibration curve of TFPI [Concentration vs Mean OD].

The precision and accuracy of standard concentrations were measured from multiple assays performed over several days.

Calibration Std. Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. deviation	% CV	% Bias	Mean Accuracy	n
2000	2002.341	8.62	0.4	0.1	100	6
1000	1003.49	8.585	0.9	0.3	100	6
500	492.711	6.272	1.3	-1.5	98.5	6
250	251.775	3.096	1.2	0.7	101	6
125	126.402	0.846	0.7	1.1	101	6
62.5	62.439	1.664	2.7	-0.1	99.9	6
31.25	31.087	0.807	2.6	-0.5	99.5	6

Fig 2: Back Calculated Standard Concentrations, accuracy and precision for TFPI in Human Sodium Citrated Plasma.

Accuracy and Precision

Intra-day accuracy and precision were determined by analyzing six replicates of QC samples at each level of concentrations within a single run. Inter-day accuracy & precision were determined by analyzing six replicates of each QC samples in six independent run on separate days.

QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. deviation	% CV	% Bias	Mean Accuracy	n
31615.225	29195.316	391.024	1.3	-7.7	92.3	6
16857.065	15365.43	351.777	2.3	-8.8	91.2	6
905.159	903.938	16.339	1.8	-0.1	99.9	6
482.159	467.238	10.691	2.3	-3.2	96.8	6
153.206	157.198	4.368	2.8	2.6	103	6

Fig 3: Intra-day precision and accuracy of QC samples.

QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. deviation	% CV	% Bias	Mean Accuracy	n
31615.225	29979.01	1638.503	5.5	-5.2	94.8	6
16857.065	15761.501	647.062	4.1	-6.5	93.5	6
905.159	918.204	35.105	3.8	1.4	101	6
482.159	475.748	16.425	3.5	-1.4	98.6	6
153.206	158.426	6.495	4.1	3.4	103	6

Fig 4: Inter-day precision and accuracy of QC samples.

Selectivity

Selectivity was performed by spike recovery. TFPI at high and low concentrations were spiked in ten source of individual serum (lipemic, haemolysed & 08 normal serums). The basal concentration was subtracted from spiked serum concentration to calculate the accuracy of spiked concentration recovery. Eight met acceptance limit at low level and all ten passed at high level.

	Spiked high Conc. (1500 pg/mL)		Spiked low Conc. (90.00 pg/mL)	
Serum ID	Reported Mean Conc. (pg/mL)	% Bias	Reported Mean Conc. (pg/mL)	% Bias
1601	1253.557	-16.4	78.144	-13.2
1605	1276.973	-14.9	79.693	-11.4
1607	1454.538	-3	84.685	-5.9
1608	1298.574	-13.4	47.867	-46.8
1610	1308.08	-12.8	79.468	-11.7
943	1338.614	-10.8	89.162	-0.9
857 (Lipemic)	1261.069	-15.9	193.185	114.7
945	1268.739	-15.4	76.4	-15.1
946	1238.533	-17.4	91.627	1.8
5% hemolyzed (944)	1255.651	-16.3	73.033	-18.8

Fig 5: Accuracy of spike recovery of TFPI in human plasma.

Stability

The absolute recoveries (high and low MC, each six sets) of TFPI reported as mean % bias were determined by comparing with calibration curve and QC samples (freshly prepared)

Theoretical TFPI Conc. (pg/mL)	Bench Top Stability at Rtafter 26 hrs.	Freeze & Thaw Cycles (Six)	Long Term Freezer Stability (after 61 days)
31615.225	-6.3	-8.4	9.9
16857.065	-9.3	-8.2	11.8

Fig 6: Stabilities of TFPI in human citrated plasma at high and low level.

Conclusion: After overcoming challenges related to commercial kit, reference standard, quality controls, robust, sensitive, precise bioanalytical assay was developed and validated to accurately quantitate human TFPI in human plasma.