Method Development and Validation of a Biomarker Analog by Hybrid LC-MS/MS with Two-Dimensional Chromatography in Human Matrices

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Introduction

The KLK1 protease is responsible for cleaving low-molecular-weight kininogen into bradykinin making it a key protein of research in blood pressure regulation. In this assay, we successfully developed and validated a method to detect a KLK1 analog utilizing hybrid LC-MS/MS and 2D-LC to gain specificity and sensitivity of the analog protein against its endogenous counterpart in human plasma.

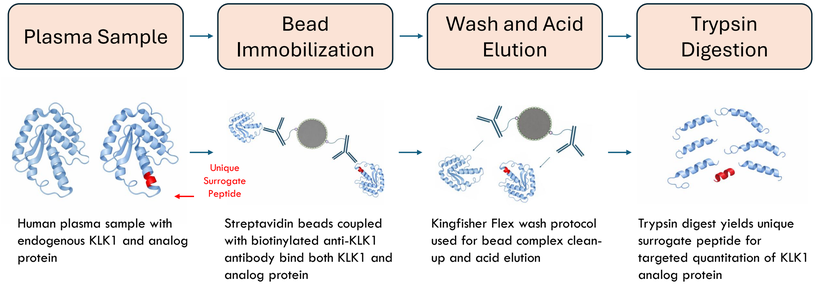
Novelty

Traditional LBA was attempted but deemed unsuitable for analysis due to inability to differentiate between KLK1 biomarker and the analog protein. Due to the nearly identical nature of KLK1 to the analog, selective capture discerning the two compounds was deemed impossible with the capture antibodies available. A novel hybrid LC-MS/MS assay has been developed and validated for the targeted quantitation of a KLK1 analog with pg/mL sensitivity allowing for PK assessments to be conducted on clinical samples. This assay showcases the ability of immunoaffinity coupled with LC-MS/MS to utilize a sub-optimal capture and surrogate peptide detection by MS/MS. This hybrid approach was developed for analyte quantitation by monitoring a surrogate peptide unique to analog analyte post tryptic digest to gain selectivity against endogenous KLK1. While the capture Ab could not differentiate between endogenous KLK1 and Analog, LC-MS/MS provided the specificity via surrogate peptide approach. An innovative 2D-LC chromatographic setup was incorporated due to the highly polar nature of the peptide allowing for chromatographic selectivity against interfering compounds and an increase in analyte sensitivity vs 1D-LC methods. Conventional 1D reverse phase chromatography showed low analyte sensitivity, retention, and unacceptable separation from interferences in the background. By utilizing the addition of trifluoroacetic acid (TFA) in the first LC phase, the highly polar peptide was able to gain retention on the C18 trap column prior to analytical flow (second phase) and improve separation from interfering compounds. The mobile phase gradient is then switched from TFA to formic acid before eluting from the analytical column to the MS. This 2D chromatography workflow allows for no MS ion suppression to occur by diverting TFA from the analytical flow while achieving separation from interfering compounds and increase in analyte signal-to-noise.

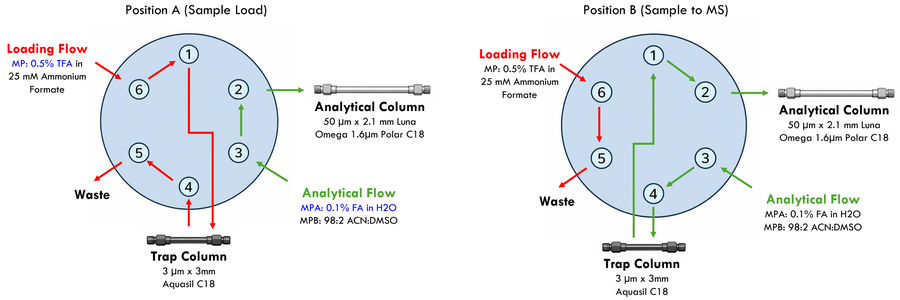
Method

Due to insufficient specificity of the capture antibody, mass spectrometry detection was incorporated for differentiation between the endogenous KLK1 and its analog. A hybrid approach was utilized with immunoprecipitation followed by a tryptic digest. Minor differences between KLK1 and the analog protein resulted in a single unique surrogate peptide suitable for analyte quantitation. The resulting surrogate peptide was of a highly polar nature causing insufficient retention on most reverse phase columns with poor peak shape. A 2D-LC approach was adopted/utilized to gain retention and aid in peak shape and sensitivity. An initial loading mobile phase consisting of TFA in water is used to retain the polar analyte on a reverse phase trap before eluting to a Luna Omega Polar C18 analytical column. The loading flow is then switched to a more MS compatible formic acid in water and ACN. The initial flow is diverted prior to analyte elution. This innovative setup allowed TFA to never be introduced to the MS while achieving retention on the trap- and-elute configuration. The method was successfully validated under ICH-M10 guidelines.

Immunoprecipitation Workflow



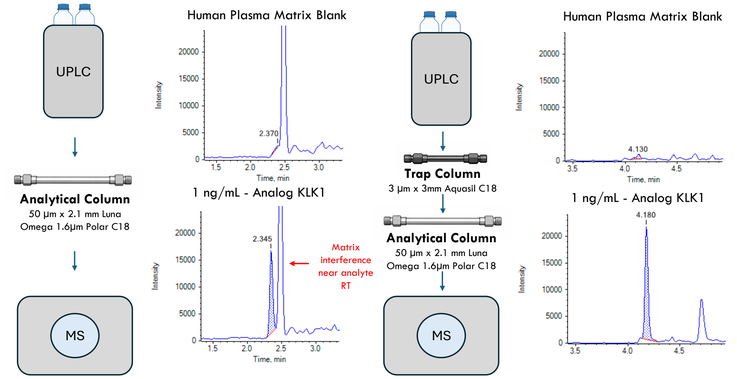
Two-Dimensional LC Flow Path



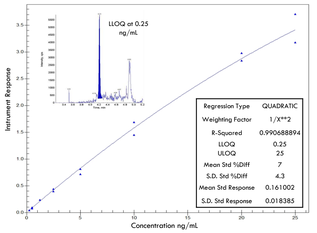
Results

This LC-MS/MS approach achieved targeted quantitation of the KLK1 analog using a unique surrogate peptide despite having a non-specific capture preventing traditional LBA approaches to be successful. A 2D-LC approach was applied to gain separation from interfering compounds and to enhance the retention of the polar surrogate peptide. This approach achieved 6x increase in signal-to-noise (S/N) from conventional 1D-LC protocols showcased by analyte area at 1 ng/mL plasma extracts increasing from S/N of 3.4 with 1D analytical flow to S/N of 21 with 2D preliminary trap flow introduced. Peak retention was improved from 2.3 min to 4.2 min gaining baseline resolution from interfering compounds present in the initial 1D analytical flow. MS/MS detection was used to gain selectivity for a unique peptide to discern between KLK1 biomarker and the analog protein. The method was developed and fully validated for a range of 0.25 - 25 ng/mL in human plasma. Accuracy and precision intra-assay and inter-assay were validated within ± 20% or ± 25% for LLOQ. Selectivity in extracted blank matrix and blank matrix with IS was assessed and shown to be clean of interferences. Matrix effect was tested in 10 individual plasma lots with 9/10 ± 20% at 0.75 and 18.8 ng/mL QC levels. Hemolytic and lipemic plasma lots were also assessed and met validation criteria at 0.75 and 18.8 ng/mL. Room temperature bench-top stability and freeze-thaw stability were validated at 15 hours and 4 cycles, respectively. All validation parameters were successfully assessed per ICH-M10 guidelines for the KLK1 analog.

Chromatography Comparison



Calibration Curve



Calibration curve of Analog KLK1 in human K2EDTA plasma from 0.25 ng/mL-25 ng/mL.

Inter-Assay Results

P&A Run	QCS 0.25 ng/mL	%Bias	QCS 0.75 ng/mL	%Bias	QCS 7.5 ng/mL	%Bias	QCS 18.75 ng/mL	%Bias
	0.250		0.750		7.50		18.8	
1	0.235	-6.0	0.655	-12.7	6.09	-18.8	19	1.1
	0.226	-9.6	0.58	-22.7	6.53	-12.9	16.8	-10.6
	0.248	-0.8	0.604	-19.5	6.97	-7.1	18.8	0
	0.232	-7.2	0.552	-26.4	5.92	-21.1	19.2	2.1
	0.256	2.4	0.612	-18.4	5.76	-23.2	18.2	-3.2
2	0.236	-5.6	0.871	16.1	6.71	-10.5	17	-9.6
	0.311	24.4	0.953	27.1	8.88	18.4	20.9	11.2
	0.291	16.4	0.861	14.8	9.38	25.1	21.2	12.8
	0.281	12.4	0.937	24.9	9.19	22.5	22.1	17.6
	0.302	20.8	0.882	17.6	8.51	13.5	20.9	11.2
	0.318	27.2	0.892	18.9	8.8	17.3	21.4	13.8
3	0.307	22.8	0.879	17.2	9.05	20.7	21.2	12.8
	0.295	18.0	0.766	2.1	8.03	7.1	17.6	-6.4
	0.223	-10.8	0.834	11.2	7.62	1.6	19.5	3.7
	0.215	-14.0	0.74	-1.3	7.93	5.7	19.2	2.1
	0.208	-16.8	0.791	5.5	7.44	-0.8	19.3	2.7
	0.27	8.0	0.687	-8.4	7.33	-2.3	19.7	4.8
	0.229	-8.4	0.759	1.2	8.22	9.6	19.5	3.7
Mean Concentration Found (ng/mL)	0.26		0.77		7.69		19.5	
	0.0368		0.129		1.17		1.53	
Inter-run %CV	14.2		16.8		15.2		7.8	
Inter-run %Bias	4		2.7		2.5		3.7	
n	18		18		18		18	

Conclusions

A hybrid LC-MS/MS assay has been developed for the targeted quantitation of a KLK1 analog with pg/mL sensitivity. A novel 2D-LC approach was utilized to achieve acceptable retention and sensitivity of a highly polar surrogate peptide for adequate analyte quantitation. This sensitive and robust method was successfully validated and used for PK bioanalysis of clinical study samples. This protocol has also been successfully validated in human breast milk and applied for mini-pig plasma analysis.