R090

Development of a HMWK Capillary Immunoblotting Assay for Characterization of Bradykinin-Mediated Disorders

Evangelia Pardali[1], Dan Sexton[2], Grit Zahn[3], Anne Lesage[4]

1. Pharvaris B.V., Leiden, The Netherlands; 2. Sexton Bio Consulting, LLC, Melrose, MA, US; 3.Globalization Partners, Munich, Germany; 4. GrayMatters Consulting, Schilde, Belgium

Introduction

- Activation of the plasma kallikrein-kinin system (KKS) results in cleavage of high molecular weight kininogen (HMWK) and production of vasodilatory kinins, such as bradykinin (BK).[1]
- BK is involved in various physiological and pathological processes, including angioedema (AE).[2] Differentiating BK-mediated from histamine-mediated AE and assessing the role of BK in the pathogenesis of other conditions, by analyzing biomarkers produced upon activation of the KKS, remains a challenge due to limitations of current analytical assays.[3]
- Cleavage of intact (i)HMWK results in the generation of BK and cleaved (c)HMWK, which can serve as a surrogate marker for BK.
- Establishment of a method to measure levels of i/cHMWK could aid in identifying and studying BK-mediated disorders.

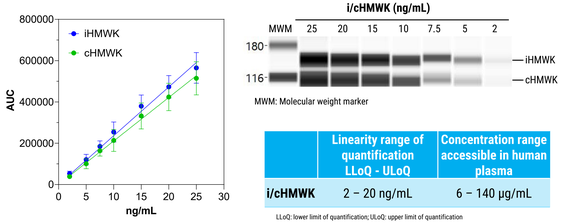
Materials and Methods

- To inhibit ex vivo activation of KKS proteases and proteolytic degradation of KKS biomarkers, a protease inhibitor (PI) cocktail was manufactured in a liquid form (Liquid PI).
- Blood samples were collected from healthy volunteers (HV) by Fidelis Research AD (Sofia, Bulgaria) in accordance with the Declaration of Helsinki and approved by The National Bioethics Committee of Medicines and Medical Devices (protocol no. FRT-19101). All participants provided their written informed consent before enrolment.
- Blood was collected using S-Monovettes (Sarstedt) with a single venipuncture with a 21G x 3/4" Safety-Multifly® needle (Sarstedt), using the aspiration technique. Following venipuncture, a small blood aliquot was collected and discarded.
- Plasma was collected into tubes containing either liquid PI or ethylenediaminetetraacetic acid (EDTA) as a control.
- A Simple Western Size (SWS) capillary immunoassay was developed under non-reducing conditions for the detection and analysis of iHMWK and cHMWK using an anti-human HMWK antibody (Abcam, Cambridge, UK) .
- Qualification of the assay was performed using Liquid PI plasma or EDTA plasma from HVs.
- Plasma samples were diluted in kininogen-deficient (KD) plasma in the presence of Liquid PI and analyzed in 2 replicates at 4 different dilutions.

Results

- Standard curves were prepared by spiking iHMWK or cHMWK in KD plasma in the presence of Liquid PI.
- For both proteins, the back-calculated concentrations of the standards met qualification criteria (**Figure 1**).

Figure 1. Standard (SD) curves of iHMWK and cHMWK in KD plasma

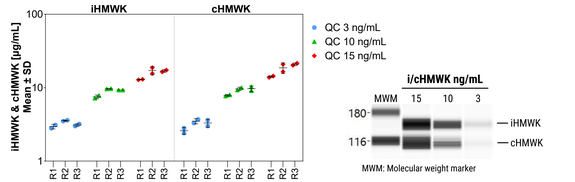


	Linearity range of quantification LLoQ - ULoQ	Concentration range accessible in human plasma
i/cHMWK	2 – 20 ng/mL	6 – 140 µg/mL

LLoQ: lower limit of quantification; ULoQ: upper limit of quantification

- To determine accuracy, recombinant iHMWK and cHMWK proteins at three different concentrations were diluted in KD plasma in presence of Liquid PI.
- Analysis of i/cHMWK quality control (QC) samples met qualification criteria for intra- and inter-run accuracy and precision. Accuracy: 80-120% of nominal concentration; Precision: Coefficient of variation (CV) <20% (**Figure 2**).

Figure 2. Analysis of quality control (QC) samples for iHMWK and cHMWK in KD plasma



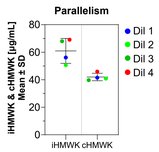
QC Sample nominal concentration	Concentration (ng/mL)				Accuracy (%)			
	iHMWK		cHMWK		iHMWK		cHMWK	
	Mean ± SD	CV%	Mean ± SD	CV%	Mean ± SD	CV%	Mean ± SD	CV%
3 ng/mL	3 ± 0	10	3 ± 0	16	107 ± 11	10	104 ± 16	16
10 ng/mL	9 ± 1	11	9.1 ± 1	11	98 ± 12	12	101 ± 11	11
15 ng/mL	16 ± 2	15.1	18 ± 3	19	87 ± 13	15	99 ± 19	19

R: run; QC: quality control; SD: Standard deviation; CV: Coefficient of variation

Results

- Assay parallelism was evaluated in EDTA plasma diluted in KD plasma at four different dilutions, in three independent experiments. Final concentration of iHMWK and cHMWK was estimated in each dilution sample.
- iHMWK and cHMWK analysis met parallelism acceptance criteria with CV <20% (**Figure 3**).

Figure 3. Parallelism for iHMWK and cHMWK in EDTA plasma

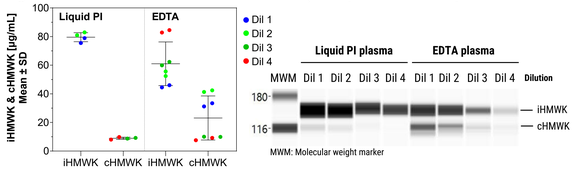


iHMWK		cHMWK	
Mean ± SD (µg/mL)	CV%	Mean ± SD (µg/mL)	CV%
61 ± 9	15	42 ± 3	7

QC: quality control; SD: Standard deviation; CV: Coefficient of variation

- Intra-run precision was analyzed in Liquid PI and EDTA control samples at 4 dilutions in duplicates and final i/cHMWK concentrations were calculated.
- Comparable levels of iHMWK and cHMWK were observed in EDTA plasma, indicating activation of KKS system.
- Significantly lower cHMWK levels were detected in Liquid PI plasma, suggesting that Liquid PI inhibited unspecific KKS activation (**Figure 4**).
- Acceptable intra-run variability was detected (CV <20%) for both iHMWK and cHMWK in Liquid PI and control EDTA plasma (**Figure 4**). However, higher variability between different dilutions was observed in EDTA plasma.

Figure 4. iHMWK and cHMWK intra-run variability in Liquid PI and EDTA plasma



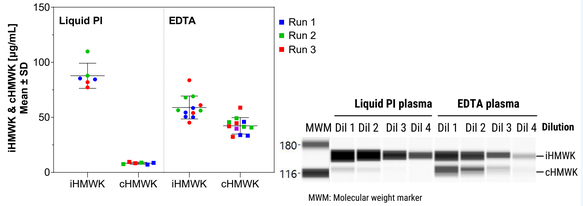
		iHMWK (µg/mL)				cHMWK (µg/mL)			
Plasma	Dil	Value 1	Value 2	Mean ± SD	%CV	Value1	Value2	Mean ± SD	%CV
Liquid PI	Dil 1	aULoQ	aULoQ	n/a	n/a	9	9	9 ± 0	1
	Dil 2	aULoQ	aULoQ	n/a	n/a	7	7	7 ± 0	3
	Dil 3	82	88	85 ± 4	5	bLLoQ	bLLoQ	n/a	n/a
	Dil 4	85	84	84.5 ± 1	1	bLLoQ	bLLoQ	n/a	n/a
EDTA	Dil 1	51	49	50 ± 1	3	35	31.6	33 ± 3	7
	Dil 2	52	50	51 ± 1	3	33	34	34 ± 1	3
	Dil 3	53	56	54 ± 2	3	9	9	9 ± 1	6
	Dil 4	61	56	58 ± 3	5	7	6	7 ± 1	7

Dil: Dilution; aULoQ: above upper limit of quantification; bLLoQ: below lower limit of quantification; QC: quality control; SD: Standard deviation; CV: Coefficient of variation

Results

- Inter-run precision was analyzed in Liquid PI and EDTA control samples at 4 dilutions in 3 independent experiments, and final i/cHMWK concentrations were calculated (**Figure 5**).
- Acceptable inter-run variability was observed (CV <20%) for both iHMWK and cHMWK in both Liquid PI plasma and control EDTA plasma (**Figure 5**).

Figure 5. iHMWK and cHMWK inter-run variability in Liquid PI and EDTA plasma



	iHMWK		cHMWK	
	Mean±SD (µg/mL)	%CV	Mean±SD (µg/mL)	%CV
Liquid PI	88 ± 11	13.0	8 ± 1	11
EDTA	59 ± 10	18	42 ± 7	18

QC: quality control; SD: Standard deviation; CV: Coefficient of variation; Dil: Dilution

Conclusions

- **Increased KKS activation resulting in cleavage of iHMWK and increased cHMWK levels was observed in EDTA plasma as compared to plasma collected in tubes with Liquid PI.**
- **Therefore, the Liquid PI cocktail was efficacious in inhibiting non-specific activation of KKS and cleavage of iHMWK, as compared to EDTA without PI.**
- **The established qualified i/cHMWK immunoassay can be used to reliably measure KKS biomarkers in human plasma.**
- **The i/cHMWK capillary immunoassay could be utilized as a key tool for identifying, studying, and managing BK-mediated diseases.**

References

1. Kaplan AP, et al. Adv Immunol. 2014;121:41-89. **2.** Maurer M, et al. Clin Rev Allergy Immunol. 2021;61:40-9. **3.** Kaplan AP, et al. Front Med (Lausanne). 2017;4:206.

COI:E.P.: employee of Pharvaris, holds stock in Pharvaris; **D.S.:** principle at Sexton Bio Consulting, LLC, consultant to Pharvaris; **G.Z.:** employee of Globalization Partners GmbH, consultant to Pharvaris, holds RSU; **A.L.:** employee of GrayMatters Consulting and consultant to Pharvaris, holds stocks/stock options in Pharvaris; advisor to Kosa Pharma. Funding statement: Funding towards this study was provided by Pharvaris.