SENSITIVE AND REPRODUCIBLE LC-MS QUANTIFICATION OF C-REACTIVE PROTEIN IN PLASMA

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INTRODUCTION

C-Reactive Protein (CRP), seen in Figure 1, is naturally synthesized in the liver and released into the bloodstream in response to inflammation. Increased plasma levels (>100 fold)[1] have been reported in patients with tissue injury or inflammatory processes such as arthritis[1,2]. This has resulted in significant interest in measuring CRP as a putative biomarker of inflammation and certain cancers. Historic LBA quantification of proteins is being replaced by LC-MS due to the many benefits it offers (e.g., multiplexing specificity, dynamic range, and fast method development times). The common bottom up approach using enzymatic digestion and analysis of resulting peptides can be a complex and time consuming workflow, with enzymatic digestion often taking 24 hours to achieve sensitive and accurate quantification.

This work describes a total workflow that can be completed in <3 hours using commercially available digestion and peptide purification kits with generic protocols for the accurate quantification of CRP from only 35 µL of plasma.

MEKLLCFLVLTSLSHAFGQTDMSRKAFVFPKESDTSYVSLKA
PLTKPLKAFTVCLHFYTELSSTRGYSIFSYATKRQDNEILIFWS
KDIGYSFTVGGSEILFEVPEVTVAPVHICTSWESASGIVEFWV
DGKPRVRKSLKKGYTVGAEASIILGQEQDSFGGNFEGSQSL
VGDIGNVNMWDFVLSPDEINTIYLGGPFSPNVLNWRALKYEV
QGEVFTKPQLWP

Figure 1. Amino acid sequence of human CRP; tryptic peptides used for quantification are high-lighted in blue

METHODS

Sample Preparation

CRP (human sequence) was spiked into rat or human plasma. Plasma samples (35 µL) were directly digested for 2 hours using the ProteinWorks eXpress Direct Digestion Kit, specifically, the 3-Step (no reduction/ alkylation) method included in the kit. Post digestion purification of signature peptides was done using the ProteinWorks µElution SPE Clean-Up Kit and included protocol.

LC-MS Conditions

LC-MS/MS quantification of resulting peptides was performed using a Waters Xevo TQ-XS triple quadrupole MS (ESI+). Chromatographic separation was achieved using an ACQUITY UPLC system with an ACQUITY UPLC HSS T3, 1.8µm , 2.1 mm x 50 mm column, at a flow rate of 0.3 mL/min using a linear gradient with 0.1% formic acid in water and acetonitrile. Signature peptides used for quantification were AFVFPK, ESDTSYVSLK, and GYSIFSYATK. MS conditions are summarized in Table 1.

Peptide	Precursor Charge State	MRM Transition	Cone Voltage (V)	Collision Energy (eV)	Product Ion ID
AFVFPK	[M+2H]2+	354.71>244.17*	35	9	[1H+]1/y2
	[M+2H]2+	354.71>219.11**	35	3	[1H+]1/b2
ESDTSYVSLK	[M+2H]2+	564.77>347.23*	35	17	[1H+]1/y3
	[M+2H]2+	564.77>696.39**	35	17	[1H+]1/y6
GYSIFSYATK	[M+2H]2+	568.78>221.09*	35	11	[1H+]1/b2
	[M+2H]2+	568.78>716.36**	35	11	[1H+]1/y6

**primary transition used for quantification and*
***confirmatory transition*

Table 1. Final MS conditions for CRP tryptic peptides, including precursors and fragment ions

RESULTS

I. Quantification of CRP in Rat and Human Plasma

A Peptide	Curve (µg/mL)	Weighting	Linear Fit (R^2)	% Accuracy Range
AFVFPK	0.025-100	$1/x^2$	0.999	95.4-103.2
ESDTSYVSLK	0.100-100	1/x	0.997	92.9-105.1
GYSIFSYATK	0.050-100	1/x	0.998	95.2-104.0

Table 2. Linear dynamic range and standard curve statistics in Rat (A) and Human (B) plasma for the CRP tryptic peptides used for quantification. Plasma samples were digested and extracted using a protein quantification kit and tryptic peptide SPE clean up kit.

B Peptide	Curve (µg/mL)	Weighting	Linear Fit (R^2)	% Accuracy Range
AFVFPK	0.050-100	$1/x^2$	0.998	93.6-104.4
ESDTSYVSLK	0.050-100	1/x	0.999	96.8-102.4

Rat Plasma QC Statistics

Peptide	CRP QC Conc. (µg/mL)	Mean Calculated Conc. (µg/mL)	Mean % Accuracy	%RSD
AFVPFK	0.075	0.071	94.3	2.16
	0.750	0.76	101.7	3.18
	7.500	7.69	102.5	1.23
	75.000	74.95	99.9	3.49
ESDTSYVSLK	0.250	0.265	106.2	2.08
	0.750	0.74	98.4	0.72
	7.500	7.21	96.1	0.97
	75.000	75.40	100.6	3.77
GYSIFSYATK	0.075	0.078	104.0	2.68
	0.750	0.73	98.0	6.15
	7.500	7.39	98.6	1.98
	75.000	74.92	99.9	5.63

Table 3. Rat plasma QC sample statistics for tryptic peptides used to quantify CRP

Human Plasma QC Statistics

Peptide	CRP Overspike Conc. (µg/mL)	CRP QC Conc. (µg/mL)	Mean Calculated Conc. (µg/mL)	Mean % Accuracy	%RSD
ESDTSYVSLK Lot #1	0.000	0.439	0.439	100.0	5.21
	0.075	0.514	0.507	98.7	1.61
	0.750	1.189	1.196	100.5	5.37
	7.500	7.939	7.781	98.0	0.73
	75.000	75.439	73.159	97.0	1.19
ESDTSYVSLK Lot #2	0.000	1.188	1.188	100.0	2.36
	0.075	1.263	1.269	100.5	2.99
	0.750	1.938	1.894	97.7	1.26
	7.500	8.688	8.295	95.5	1.40
	75.000	76.188	74.171	97.3	1.36
ESDTSYVSLK Lot #3	0.000	1.736	1.736	100.0	1.19
	0.075	1.811	1.741	96.1	2.74
	0.750	2.486	2.267	91.2	2.75
	7.500	9.236	8.346	90.4	0.24
	75.000	76.736	70.943	92.4	3.73
ESDTSYVSLK Lot #4	0.000	16.840	16.840	100.0	2.15
	0.075	16.915	16.827	99.5	4.51
	0.750	17.590	16.853	95.8	2.34
	7.500	24.340	22.490	92.4	5.84
	75.000	91.840	80.737	87.9	2.49

Table 4. Human Plasma QC sample statistics for the tryptic peptide, ESDTSYVSLK, used to quantify CRP in four lots of human plasma

Endogenous CRP Concentrations

Peptide	Plasma	Mean Calculated Endogenous Conc. (µg/mL)	Mean Calculated Endogenous Conc. (µg/mL)
		354>244	**354>219**
AFVPFK	Lot #1	0.387	0.381
	Lot #2	1.167	1.145
	Lot #3	1.867	1.89
	Lot #4	18.128	18.273
		364>347	**364>696**
ESDTSYVSLK	Lot #1	0.439	0.666
	Lot #2	1.188	1.145
	Lot #3	1.736	1.952
	Lot #4	16.84	17.015

Table 5. Calculated endogenous CRP concentrations in four lots of human plasma using the AFV and ESD tryptic peptides

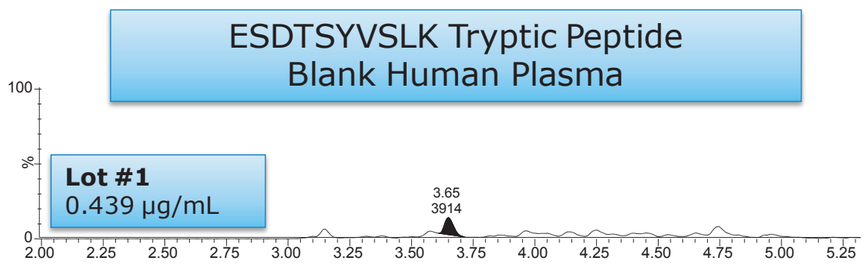

Figure 3. Representative chromatograms highlighting endogenous CRP concentrations in four lots of human plasma represented by the ESD tryptic peptide

DISCUSSION

Early detection of elevated CRP levels could be imperative for proper treatment or prevention of diseases, such as arthritis or heart disease. Availability of analytical methods that can detect low levels and differentiate between small concentration differences of CRP can facilitate early detection. Using commercially available protein digestion and peptide purification kits and protocols, successful quantification of endogenous CRP in plasma was achieved.[3]

- Through direct digestion (no affinity purification) of 35 µL of plasma and subsequent peptide purification, quantification limits between 0.025-0.1 µg/mL (1-4 nM) were readily achieved for the three signature tryptic peptides of human CRP (Table 2). In addition, standard curves were linear over 4 orders of magnitude.
- Using mixed-mode SPE and generic protocol resulted in >90% recovery for the three peptides.
- Total sample preparation time, including SPE, was <3 hours.
- The accuracy and precision for CRP (human sequence) quantified in rat (1 lot) and human plasma (4 lots) was excellent with accuracies ranging from 88-106% and RSDs <6%. CRP QC statistics in rat and human plasma are highlighted in Tables 3 and 4, and illustrated in Figure 2, Panels A and B, respectively.
- Representative chromatograms illustrating the endogenous CRP levels in 4 lots of human plasma are highlighted in Figure 3.
- Calculated endogenous CRP concentrations in human plasma, derived from either AFV or ESD tryptic peptides, were within 10% agreement (Table 5). Confirmatory transitions for each peptide were used to verify endogenous concentrations of each plasma lot.

CONCLUSION

Using a generic kit-based approach (with simple step-wise protocols and standardized, pre-measured reagents) for digestion and subsequent peptide purification, accurate CRP quantification in plasma was achieved. The specificity, sensitivity (0.025 µg/mL), broad linear dynamic range, and exceptional reproducibility (RSD <6%) of the method described reliably measures low endogenous and elevated levels of CRP in plasma that would be expected in diseased populations.

References

1. Kun, E., Wu, J., Karl, J., Liao, H., Zolg, W., Guild, B. (2004)Quantification of c-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and ^{13}C-labeled peptide standards. Proteomics (Issue 4), pages 1175-1186.
2. Allin. K.H., Nordestgaard, B.G., (2011) Elevated c-reactive protein in the diagnosis, prognosis, and cause of cancer. Critical reviews in clinical laboratory sciences. Volume 48 (Issue 4), pages 155-170.
3. Waters Application Note: (720005819EN) Sensitive and Reproducible LC-MS Quantification of C-Reactive Protein in Plasma: A Potential Biomarker of Inflammation

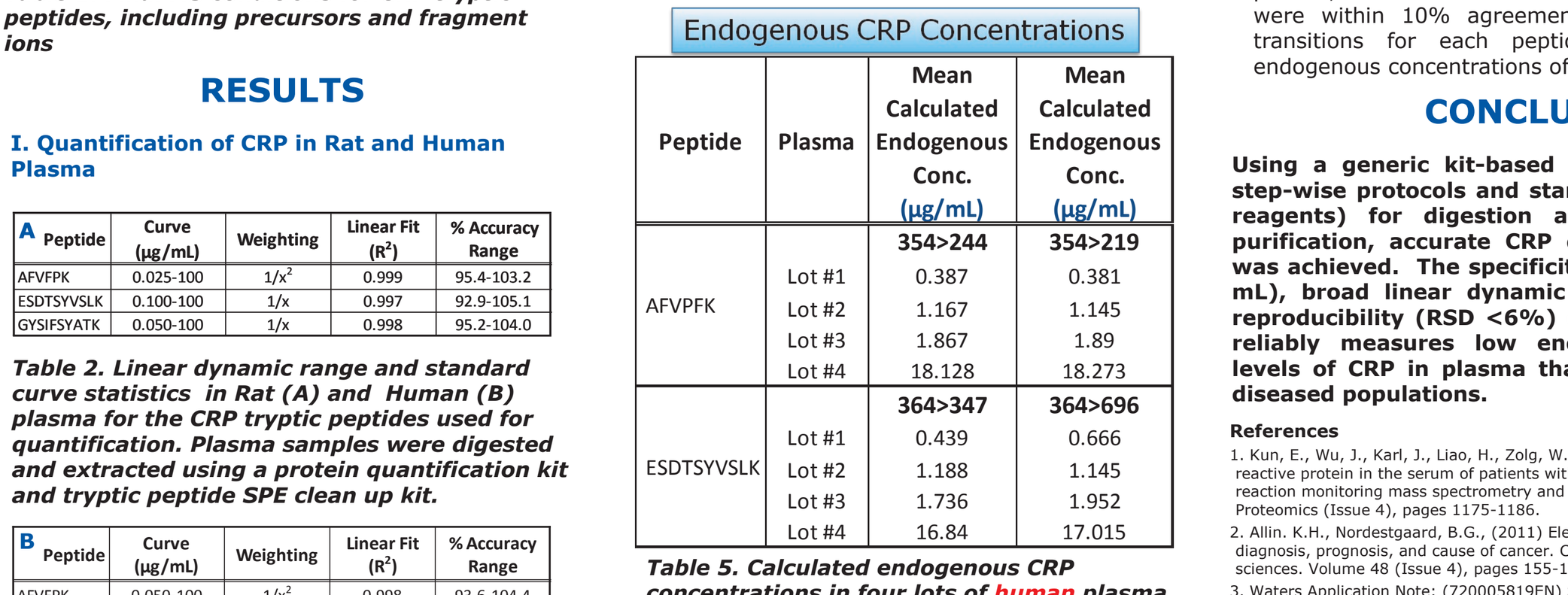

Figure 2. Representative QC chromatograms of CRP in rat (A) and human (B) plasma, digested and extracted using a protein quantification digestion and peptide purification kit

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