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

Paula Orens, Mary Lame, Steven Calciano, and Erin Chambers
Waters Corporation 34 Maple Street Milford, MA 01757

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INTRODUCTION

In the past decade, identification and quantification of biomarkers has become an integral part of drug discovery and clinical research. 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 analytical quantification of proteins is being replaced by LC-MS due to the many benefits it offers (e.g., multiplexing selectivity, 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 highlighted 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

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

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

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.

Rat Plasma QC Statistics

Peptide	CRP QC Concentration (µg/mL)	Calculated Concentration (µg/mL)	Mean % Accuracy	%RSD
AFVPFK	0.075	0.071	94.3	2.16
	0.750	0.763	101.7	3.18
	7.500	7.691	102.5	1.23
	75.000	74.946	99.9	3.49
ESDTSYVSLK	0.250	0.265	106.2	2.08
	0.750	0.738	98.4	0.72
	7.500	7.210	96.1	0.97
	75.000	75.399	100.6	3.77
GYSIFSYATK	0.075	0.078	104.0	2.68
	0.750	0.735	98.0	6.15
	7.500	7.394	98.6	1.98
	75.000	74.918	99.9	5.63

Table 3. Rat plasma QC sample statistics for the tryptic peptides, AFVPFK, ESDTSYVSLK, and GYSIFSYATK, used to quantify CRP

Human Plasma QC Statistics

Peptide	CRP Overspike Concentration (µg/mL)	CRP QC Concentration (µg/mL)	Calculated Concentration (µg/mL)	Mean % Accuracy	%RSD
ESDTSYVSLK Lot #1	0.000	0.439	0.439	100.00	5.21
	0.075	0.514	0.507	98.67	1.61
	0.750	1.189	1.196	100.53	5.37
	7.500	7.939	7.781	98.00	0.73
	75.000	75.439	73.159	97.00	1.19
ESDTSYVSLK Lot #2	0.000	1.188	1.188	100.00	2.36
	0.075	1.263	1.269	100.50	2.99
	0.750	1.938	1.894	97.73	1.26
	7.500	8.688	8.295	95.50	1.40
	75.000	76.188	74.171	97.33	1.36
ESDTSYVSLK Lot #3	0.000	1.736	1.736	100.00	1.19
	0.075	1.811	1.741	96.13	2.74
	0.750	2.486	2.267	91.20	2.75
	7.500	9.236	8.346	90.37	0.24
	75.000	76.736	70.943	92.43	3.73
ESDTSYVSLK Lot #4	0.000	16.840	16.840	100.00	2.15
	0.075	16.915	16.827	99.50	4.51
	0.750	17.590	16.853	95.83	2.34
	7.500	24.340	22.490	92.40	5.84
	75.000	91.840	80.737	87.90	2.49

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

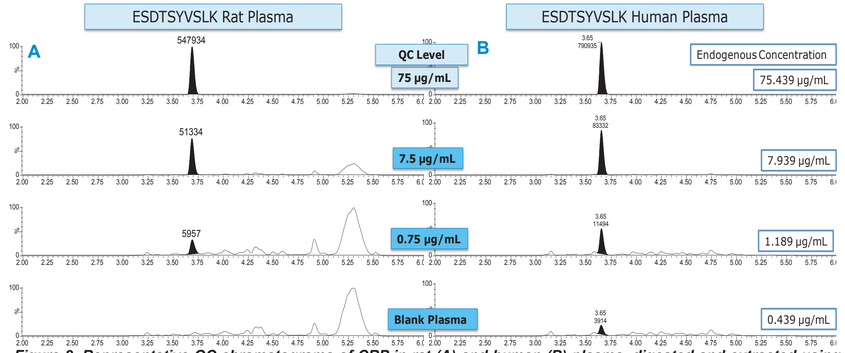

Figure 2. Representative QC chromatograms of CRP in rat (A) and human (B) plasma, digested and extracted using a protein quantification digestion and peptide purification kit

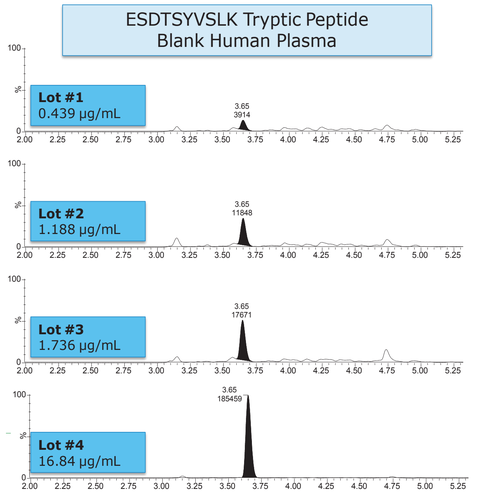

Figure 3. Representative chromatograms highlighting endogenous CRP concentrations in four lots of human plasma represented by the ESD tryptic peptide

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

DISCUSSION

In drug discovery and clinical research, availability of analytical methods that can accurately detect, quantify, and differentiate between small concentration differences of biomarkers are in high demand. In this work, successful quantification of the biomarker, CRP, in plasma was achieved using commercially available protein digestion and peptide purification kits.[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 selectivity, analytical 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.

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.
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