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Development of simplified quantitative method for tryptic digested C-reactive protein by using online SPE coupled to triple quadrupole mass spectrometer

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Introduction

Method development for accurate quantification of biomarker protein is very important step for the researchers in pharmaceutical industries and clinical fields. Mass spectrometry based quantitative proteomics approach shows a big potential to be the substitution of ELISA due to its precision, accuracy and multiplexing capability. In order to achieve the required sensitivity and selectivity, nano scale liquid chromatography mass spectrometry or sample preparation method including depletion of dominant protein and/or enrichment of target protein are frequently used. In this study, we tried to develop simple and high sensitive quantification method for C reactive protein (CRP), the level of which rise as a result of inflammation etc., by using online SPE coupled to triple quadrupole mass spectrometer without any complicated sample enrichment and depletion.

Figure 1. LCMS-8060 triple quadrupole mass spectrometer

Methods and Materials

Three tryptic peptides AFVFPK, ESDTSYVSLK, and GYSIFSYATK of human CRP are analyzed using a triple quadrupole mass spectrometer LCMS-8060 (Shimadzu Corporation, Japan) coupled with conventional flow liquid chromatography (Nexera UHPLC with online SPE system; Shimadzu Corporation, Japan). Pooled human plasma were reduced, alkylated, and digested by trypsin. While tryptic digests from biological samples were generally analyzed after manual desalting and lyophilizing steps, samples including highly concentrated urea were directly injected and desalting step was performed in on-line SPE. Sensitivity, repeatability, accuracy, and loading capacity were evaluated by human plasma digests spiked with synthetic stable isotope peptide and non-labeled peptide.

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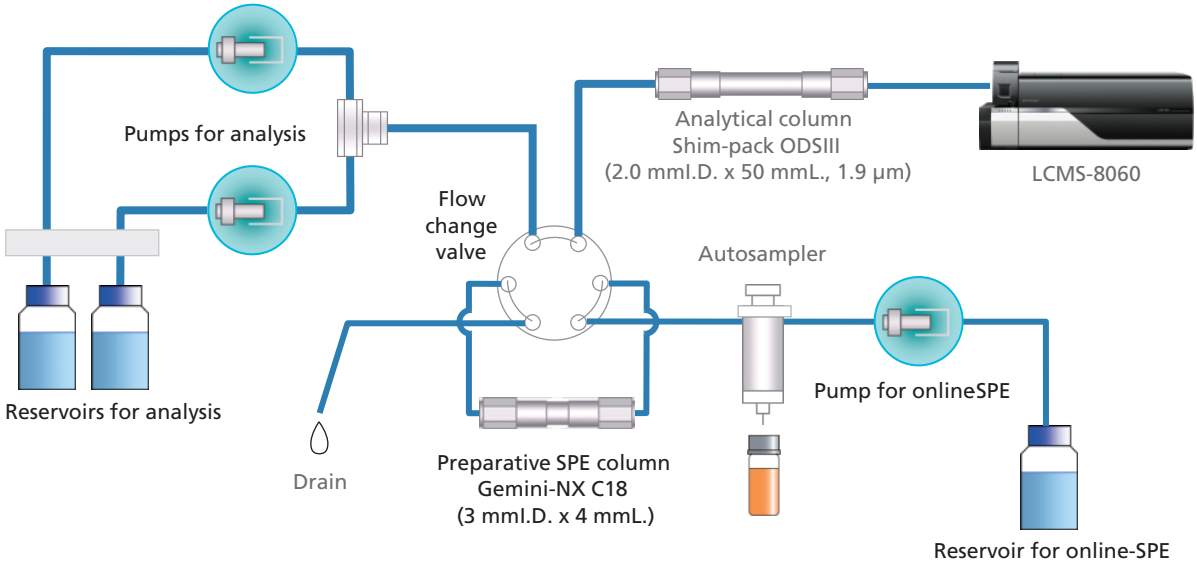

Figure 2. Flow diagram for online SPE system

Result

Method development

A simplified quantitative protocol for trypsin digest of human CRP was developed. On-line desalting condition and gradient program were optimized using standard peptide and tryptic digest from human plasma. As a result, de-saltation and chromatographic separation were performed successfully in five minutes for each sample.

We also assessed loading capacity in on-line SPE system taking into account of many of interferences and matrix effects because total plasma contains 70 g/L protein mass whereas normal CRP level is considered less than 3 mg/L. It was optimized to 10 µg protein per injection due to matrix effects.

UHPLC conditions (Nexera X2 with online-SPE system)	
Mobile phase A	: 0.1 % formic acid in water
Mobile phase B	: Acetonitrile
Solvent for sample loading	: 0.1% formic acid in water
Flow rate	: 0.4 mL/min (for analytical column), 1.0 mL/min (for sample loading)
Gradient program	: 10%B (0.00-1.00 min) > 20%B (4.50 min) > 95%B (4.6-4.99 min) > 10%B (5.00 min)
Injection vol.	: 50 µL
Column temperature	: 40 °C
MS conditions (LCMS-8060)	
Ionization	: ESI, Positive dMRM mode

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Table 1. MRM transitions for tryptic peptide of human CRP

peptide	type	transition	CE
AFVFPK	Qualifier	354.70>490.3	-12
	Confirmation	354.70>391.25	-15
	Confirmation	354.70>147.1	-23
	Confirmation	354.70>637.35	-15
ESDTSYVSLK	Qualifier	564.75>347.25	-18
	Confirmation	564.75>912.45	-21
	Confirmation	564.75>696.4	-21
	Confirmation	564.75>609.35	-19
GYSIFSYATK	Qualifier	568.80>916.5	-19
	Confirmation	568.80>829.45	-19
	Confirmation	568.80>716.35	-20
	Confirmation	568.80>248.15	-29

peptide	type	transition	CE
AFVFPK (ISTD)	Qualifier	358.70>498.3	-12
	Confirmation	358.70>399.25	-15
	Confirmation	358.70>155.15	-23
	Confirmation	358.70>645.4	-15
ESDTSYVSLK (ISTD)	Qualifier	568.80>355.25	-18
	Confirmation	568.80>920.50	-21
	Confirmation	568.80>704.40	-21
	Confirmation	568.80>617.35	-19
GYSIFSYATK (ISTD)	Qualifier	572.80>924.5	-19
	Confirmation	572.80>837.45	-19
	Confirmation	572.80>724.4	-20
	Confirmation	572.80>256.15	-29

Sample preparation

Plasma proteins were denatured with 6M Urea. Denatured proteins were reduced and alkylated with 2mM TCEP and 5mM IAA, respectively, and digested with trypsin (Enz : Protein ratio = 1 : 50). The human plasma digests quenched with 0.1% formic acid were directly injected onto online-SPE system without de-saltation.

online SPE

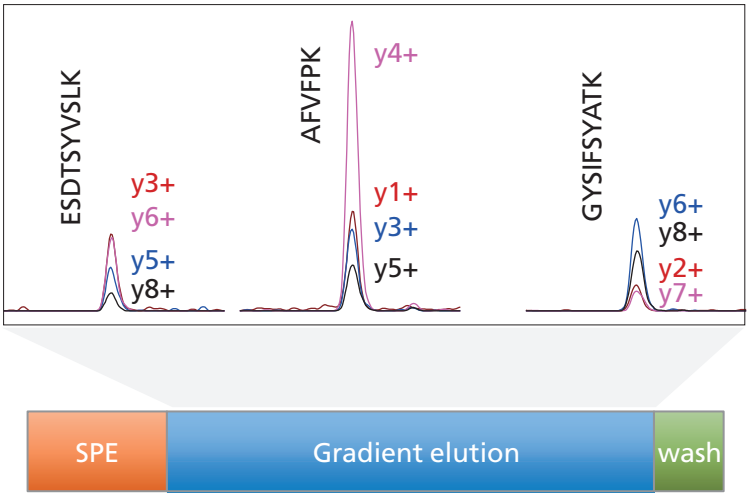

Figure 3. Time program for online-SPE analysis

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Peptide sensitivity in solvent

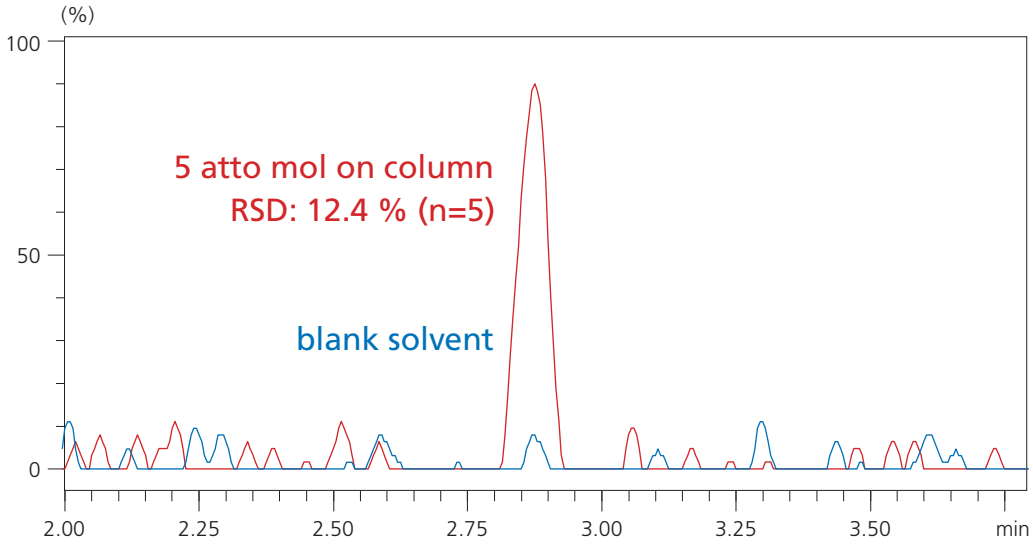

Figure 4. Representative chromatogram of trace amount of synthetic peptide AFVFPK in neat solution.

Loading capability

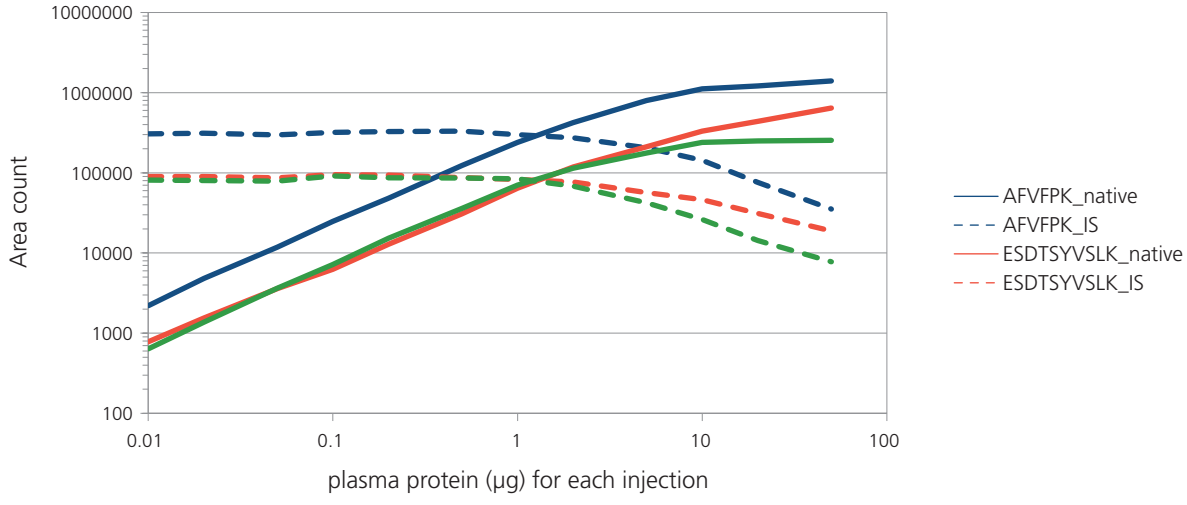

Figure 5. The plot of signal response of each target peptide (native) and their isotopic labeled internal standard peptides (ISs) as a function of the injected plasma protein amount for each analysis.
Aliquot of target peptides were spiked into plasma. The amount of ISs on column were fixed for each injection.

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Sensitivity comparison of LCMS-8060 and LCMS-8050

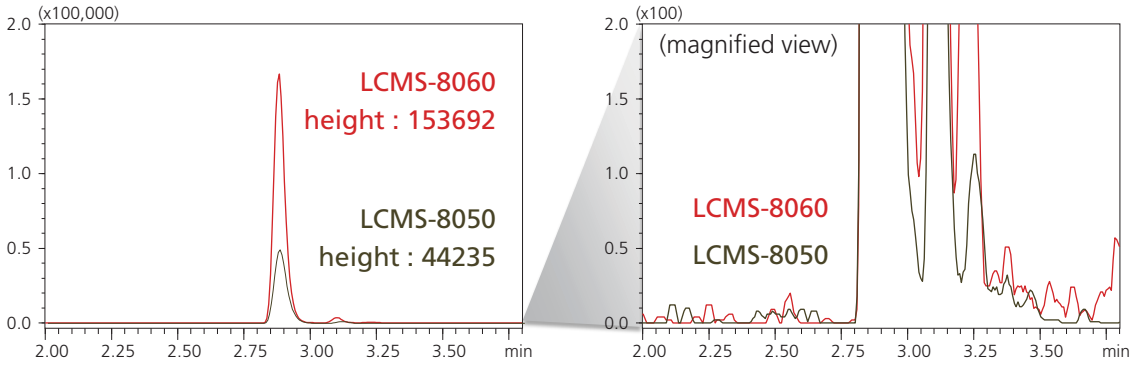

Figure 6. Chromatograms of 1.61 mg/L AFVFPK. The ratio of signal response was greater than three, but significant increasing of noise was not observed as shown in magnified view.

Analysis of hCRP in pooled human plasma.

To assess quantitative capability, tryptic peptides of hCRP in pooled human plasma were analyzed. Concentration of labeled ISTD was set at risk level of hCRP.

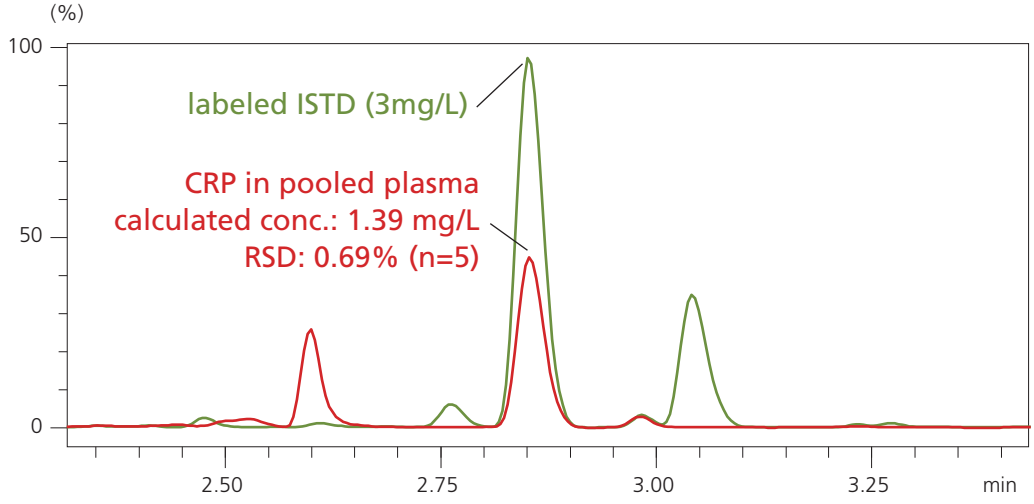

Figure 7. Tryptic peptide of hCRP (AFVFPK) in pooled human plasma.

Qualitative analysis of peptide in plasma

The linearity range for quantification was determined by calibration curve using matrix matched stable isotope peptides sample. As a result, quantification for 5 pmol/L (8.05 µg/L) in plasma was achieved.

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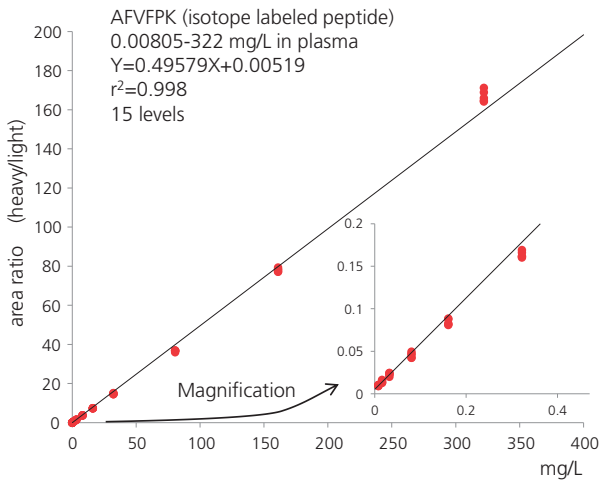

Lv	Spiked conc		Calculated conc	Accuracy	RSD
	(nmol/L)	(mg/L)	(mg/L)	(%)	(%)
1	0.005	0.00805	0.00929	115	7.1
2	0.01	0.0161	0.0181	112	8.2
3	0.02	0.0322	0.0356	111	5.2
4	0.05	0.0805	0.0813	101	3.9
5	0.1	0.161	0.159	99	2.2
6	0.2	0.322	0.320	99	1.2
7	0.5	0.805	0.785	97	0.9
8	1	1.61	1.53	95	0.6
9	2	3.22	3.01	93	0.7
10	5	8.05	7.48	93	0.8
11	10	16.1	14.8	92	0.3
12	20	32.2	29.8	93	1.0
13	50	80.5	73.9	92	0.5
14	100	161	157	98	0.6
15	200	322	337	105	0.9

Figure 8. Summery for quantitative analysis of tryptic peptide of hCRP in plasma

Conclusion

- The combination of online SPE and newly developed high sensitive triple quadrupole mass spectrometer can achieve high sensitivity and high throughput analysis.

The product and application are Research Use Only. Not for use in human clinical diagnostics or in vitro diagnostic procedures.

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