Automated High-Throughput Analysis of Proteins and Peptides in Blood-Based Matrices Using Immunoaffinity Purification and Ultrafast SPE/MS/MS

Application Note

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Introduction

Measuring biomarkers in plasma is challenging due to the complexity of the matrix. Currently, immunoaffinity purification followed by LC/MS/MS is often employed to isolate and quantify proteins or peptides. However, both steps are limited by inefficiency; immunoaffinity purification is labor intensive, while LC/MS/MS analysis requires minutes per sample. Automation of the immunoaffinity purification step, allowing for simultaneous preparation of 96 samples, followed by online SPE/MS/MS analysis at a rate of seconds per sample could greatly improve the throughput of this workflow. We investigated the feasibility of using such an automated high-throughput workflow to analyze both proteins and peptides from blood-based matrices.

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Experimental

The RapidFire/MS/MS system consists of the following modules: an Agilent RapidFire 360, an Agilent 6460 Triple Quadrupole Mass Spectrometer, and MassHunter Qualitative Analysis B.04.00 and RapidFire Integrator Software.

The Agilent Bravo Platform for AssayMAP consisted of the following modules: the Bravo Workstation for AssayMAP, AssayMAP Streptavidin (SA-W) Cartridges, and VWorks Automation Control Software with the AssayMAP Affinity Purification form.

Samples were analyzed at a rate < 15 seconds per sample using the conditions shown in Table 1. FLAG®-peptideC and its internal standard were monitored simultaneously in all experiments.

Chemicals and reagents

The analyte FLAG-peptideC and its stable-labeled isotope internal standard FLAG-peptideC-(^{13}C5, ^{15}N, D8) were synthesized by American Peptide Company, Sunnyvale, CA. Rat serum was purchased from Xenotech, Lenexa, KS. All other solvents and reagents were purchased from Sigma-Aldrich, St. Louis, MO.

Sample preparation

Calibration standards were prepared by spiking buffer or rat serum with FLAG-peptideC to final concentrations ranging from 7.8 nM to 1 µM. The samples were then purified using the Agilent Bravo Platform for AssayMAP with AssayMAP streptavidin (SA-W) cartridges (p/n G5496-60010) to immobilize the FLAG antibody that binds the analyte of interest. Eluted samples were diluted 1:1 with MeOH containing 0.1% formic acid.

Table 1. Bravo Platform for AssayMAP and RapidFire conditions.

Bravo Platform for AssayMAP conditions						
Priming	100 µL at 300 µL/min; 1 × PBS (pH = 7.4)					
Equilibration	50 µL at 25 µL/min; 1 × PBS (pH = 7.4)					
Ligand loading	50 µL at 5 µL/min; 5, 15, or 50 µg biotin anti-FLAG antibody in 1 × PBS (pH = 7.4)					
Sample loading	50 µL at 2 µL/min; FLAG-peptideC in 1 x PBS (pH = 7.4), or Rat serum					
Sample chase	5 µL at 2 µL/min; 1 × PBS (pH = 7.4)					
Washing	50 µL at 25 µL/min; 1 × PBS (pH = 7.4)					
Elution	50 µL at 5 µL/min; 12 mM glycine + 100 mM NaCl					
RapidFire conditions						
Buffer A	Water with 0.1% formic acid; 1.5 mL/min flow rate					
Buffer B	90 % acetonitrile with 0.1 % formic acid; 1.25 mL/min flow rate					
Injection volume	10 µL					
SPE cartridge	Agilent RapidFire cartridge E (reversed-phase C8 chemistry, G9207A)					
RF state 1	sip sensor					
RF state 2	7,000 ms					
RF state 3	5,000 ms					
RF state 4	500 ms					
Triple quadrupole conditions						
Gas temperature	250 °C					
Gas flow	11 L/min					
Nebulizer	30 psi					
Sheath gas temperature	250 °C					
Sheath gas flow	11 L/min					
Nozzle voltage	1,500 V					
Capillary voltage	5,500 V					
	Q1	Q3	Dwell	Fragmentor	CE	CAV
IS	1,008.2	1,034.7	50	380	20	3
Quantifier	1,004.7	1,290.4	50	380	15	2
Qualifier	1,004.7	1,029.9	50	380	20	3

Data analysis

RapidFire Integrator software was used for peak integration. The quantifier ion AUC of FLAG-peptide C was normalized by the AUC of the internal standard. The data was subjected to linear regression with 1/x weighting.

Results and Discussion

The loading capacity of the Agilent AssayMAP SA-W cartridges is 75 µg of biotinylated antibody. The following amounts of antibody were immobilized on SA-W cartridges: 5, 15, and 50 µg. An 8-point serial dilution of FLAG-peptideC in rat serum was generated for each antibody concentration. The dilutions were loaded onto the cartridges, washed, and eluted. The eluted samples were then diluted and run on the Agilent RapidFire High-throughput Mass Spectrometry System (RapidFire/MS). Comparable results were obtained using 5, 15, and 50 µg of antibody with up to 250 nM of analyte, suggesting it is not necessary to saturate the cartridges (Figure 1).

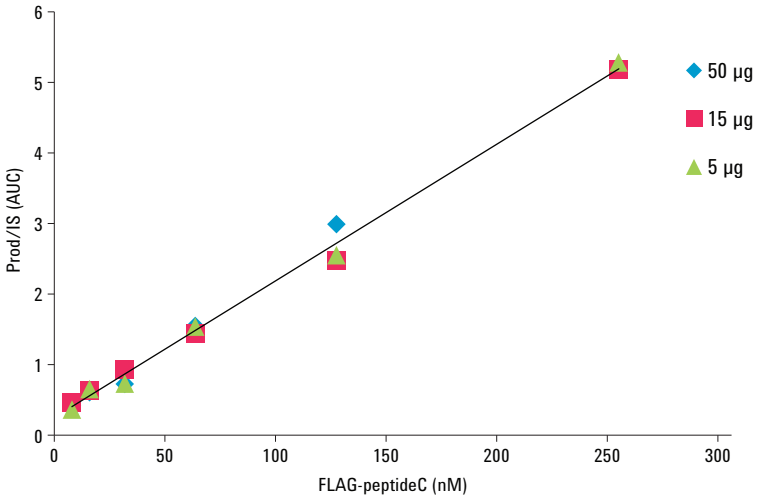

Figure 1. Loading capacity of cartridge.

Next, 5 µg of antibody was immobilized on the SA-W cartridges. A 6-point serial dilution of FLAG-peptideC in rat serum was generated. The dilutions were loaded onto the cartridges, washed, and eluted. The standard curves were identical suggesting that recovery efficiency was unaffected by sample matrix and that no significant ion suppression occurred in the serum standard curve (Figure 2). The same cartridges were reused. Even after multiple uses of the cartridge, data quality remained unaffected.

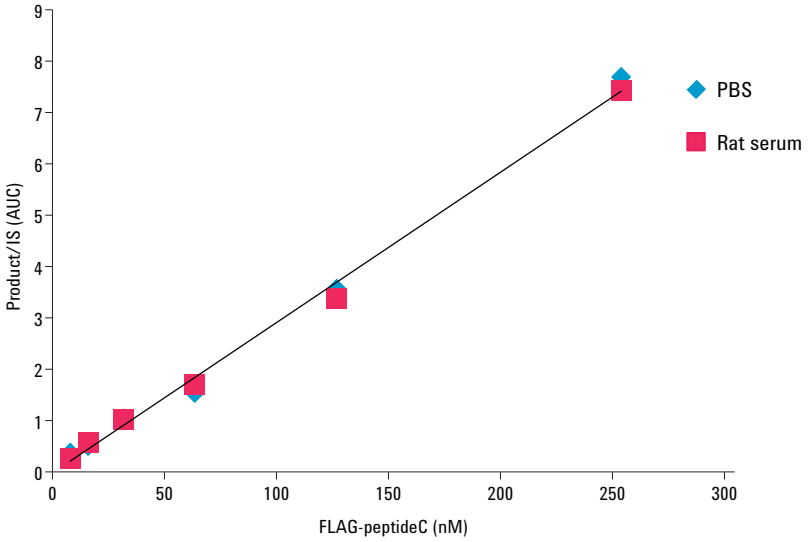

Figure 2. Recovery efficiency is unaffected by matrix.

Standard curves of FLAG-peptideC in rat serum were analyzed to obtain precision and accuracy values on the Bravo Platform for AssayMAP coupled with the RapidFire/MS. The samples were analyzed in replicates of four. Standard curves had excellent linearity within the measured range, with an R^2 value greater than 0.995 (Figure 3). Accuracies determined were within 15 % and coefficient of variation values were all less than 15 % for concentrations within the measured range (Table 1).

These results were presented at the 2012 American Society of Mass Spectrometry (ASMS) meeting.[1]

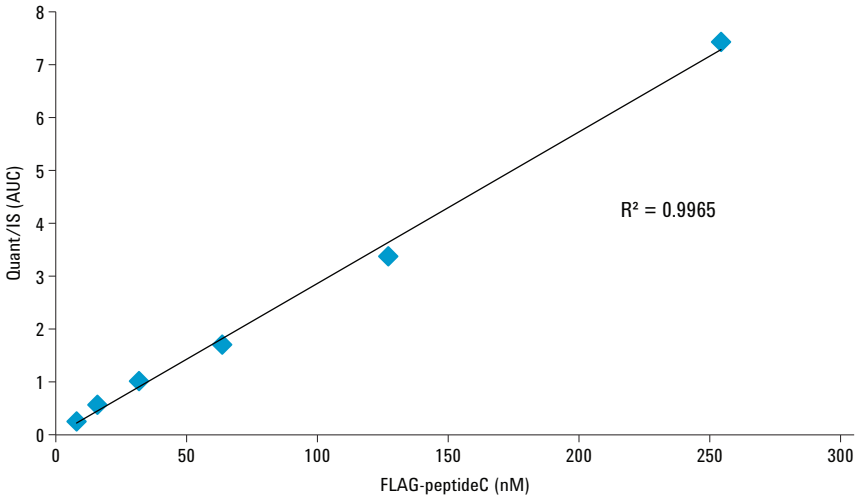

Figure 3. Representative standard curve for FLAG-peptideC spiked into serum.

Conclusions

Automated, high-throughput immunoaffinity extraction coupled with ultrafast SPE/MS/MS allows for the rapid analysis of FLAG-peptideC in rat serum. Total analysis time from serum to results for 96 samples is just over 1 hour. The immunoaffinity cartridges were optimized for efficient use of capture antibody and could be reused multiple times. Immunoaffinity purification showed equivalent results between samples prepared in PBS or serum suggesting that recovery efficiency was unaffected by the sample matrix. The quantitation of FLAG-peptideC across a concentration range of 7.8 to 250 nM produced good linearity, accuracy, and precision results. This methodology may be useful for the analysis of similar biomolecules in complex blood-based matrices.

Table 1. Intraday precision and accuracy for FLAG-peptideC in serum.

FLAG-peptideC (nM)	Intraday % accuracy (n=4)	Intraday % precision (n=4)
7.8	85.3	6.6
15.6	114.2	14.0
31.2	108.6	2.4
62.5	94.2	7.2
125	95.1	8.6
250	105.9	8.1

Reference

1. Michelle V. Romm, *et al.*; Automated High-Throughput Analysis of Proteins/Peptides in Blood-based Matrices Combining Immunoaffinity Purification and Ultra-fast SPE/MS/MS. Presented at the 59th ASMS Conference on Mass Spectrometry and Allied Topics, **2012**, Vancouver, BC.

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