High-Throughput SISCAPA-Based Peptide Quantitation Using an Agilent RapidFire High-Throughput Mass Spectrometry System

Application Note

Authors

Leigh Anderson
SISCAPA Assay Technologies
Rockville, MD USA

Morteza Razavi, Matt Pope, and Terry Pearson
University of Victoria
Biochemistry Department
Canada

Lauren Frick, Christine Miller, and William LaMarr
Agilent Technologies, Inc.
Wakefield, MA USA

Abstract

A high-throughput SISCAPA workflow has been developed that uses RapidFire/MS technology to enable sensitive quantitation of peptides from plasma digests with a sample cycle time of approximately 10 seconds. The optimized system includes capture of target proteotypic peptides using rigorously selected, high affinity antipeptide monoclonal antibodies and reduction of background peptides using a novel treatment with magnetic bead immunoadsorbents. The method can be multiplexed, thus facilitating analysis of large sample sets for biomarker analysis in a fraction of the time of other methods.

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Introduction

Mass spectrometry (MS) is increasingly used to measure proteins in biological fluids, where it provides an effective method for the reproducible quantitation of biomarkers.[1] The limited sensitivity of such assays (approximate µg/mL levels in unfractionated digests, even with nanospray ionization) and the low throughput (typically 30 – 45 min/sample) associated with peptide chromatography represent major limitations in the validation of candidate biomarkers. Effective validation of biomarker proteins in plasma, of which thousands have been reported, requires analysis of 1,000 – 2,000 samples,[3] often with sensitivities typically in the sub-ng/mL range.[4,5] Improvements in throughput and the sensitivity necessary to generate such data are, therefore, critical for biomarker translation, and might also allow MS to replace immunoassays for the routine measurement of protein markers in laboratories.[3]

The method known as stable isotope standards and capture by antipeptide antibodies (SISCAPA) has been developed to enrich low abundance peptides from complex mixtures such as trypsin-digested human plasma or serum.[6] While SISCAPA significantly improves the sensitivity of triple quadrupole MS assays, the workflow has usually employed peptide separation chromatography upstream of MS analysis to reduce interferences from remaining nonspecific peptide background, with a cycle of approximately 5.5 minutes using standard flow (approximately 1.2 mL/min) HPLC.

This application note describes a SISCAPA-RapidFire/MS workflow that quantifies multiple peptide analytes with a cycle time of approximately 10 seconds/sample. The optimized methods include an "addition only" protocol to facilitate the efficient and reproducible tryptic digestion of plasma samples, and the use of crude tryptic digests, selective capture of target proteotypic peptides using very high affinity antipeptide rabbit monoclonal antibodies (RabMabs), reduction of background peptides to extremely low levels using novel treatment of magnetic bead immunoadsorbents, and the ultra-fast injection of samples into an Agilent 6490 Series Triple Quadrupole Mass Spectrometer using RapidFire/MS technology.

Experimental

SISCAPA

Protein analytes of varying abundance in human plasma were selected to test the general utility of the methods described in this application note. Peptides that occur in a single protein encoded within the human genome and that yield several, strong MRM transitions in a triple quadrupole mass spectrometer were selected according to criteria previously described.[6] RabMAbs specific for surrogate tryptic peptides were made by Epitomics Inc. (Burlingame, CA). Tryptic digestion involved an "addition only" method to facilitate efficient and reproducible results. This method used an optimized washing procedure to remove both lipid impurities and peptides that bind nonspecifically to the magnetic immunoadsorbent beads, allowing the trypsin-digested plasma to be used directly.

The SISCAPA assay shown in Figure 1 used a trypsin-digest from 30 µL of plasma for the antibody capture of peptides. All procedures were performed using a KingFisher 96 bead-handling robot (Thermo Electron Corporation, Vantaa, Finland).

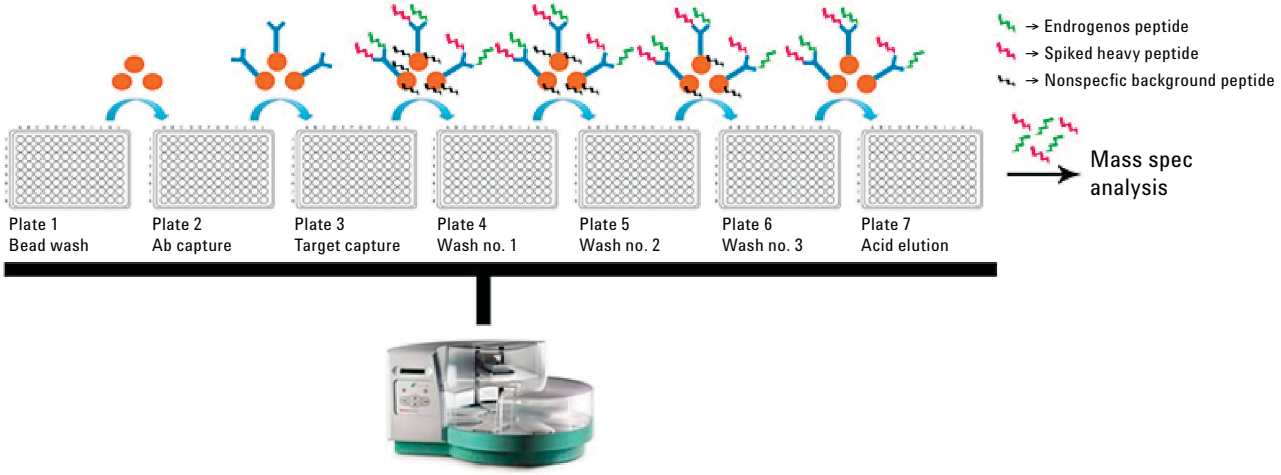

Figure1. SISPCAPA assay procedure using the KingFisher 96-bead handling robot.

A series of KingFisher plates was first prepared as shown in Table 1. Briefly, the washed beads were transferred through the antibody plate where they were allowed to capture 1 µg of the relevant antibodies. The bead-antibody complex was then transferred to the plasma digest plate to capture the target peptides. The nonspecifically bound peptides were washed away by the optimized wash buffer in the Wash Plates and finally the captured peptides were eluted and equally split for analysis using either an Agilent RapidFire 300 coupled to an Agilent 6490 Triple Quadrupole Mass Spectrometer or an Agilent 6490 Triple Quadrupole LC/MS System.

Table 1. KingFisher plates used in the RapidFire/MS, SISCAPA-based assay.

Plate number	Description
Plate 1	A bead wash plate containing 1.43 µL/well of MyOne Protein G Dynabeads (Invitrogen-Dynal; custom made 1.0 µm diameter; low peptide binding) brought up to 200 µL/well in PBS/0.03 % of the zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS).
Plate 2	An antibody capture plate containing 1.0 µg/well of desired antibody brought up to a final volume of 100 µL/well in PBS/0.03 % CHAPS.
Plate 3	A peptide capture plate containing the trypsin digest of 30 µL plasma. The corresponding Stable Isotope Standard (SIS) peptides were added at this stage at 500 fmol/well.
Plates 4 and 5	Two wash plates (no. 1 and no. 2) containing 250 µL/well of PBS/0.03 % CHAPS.
Plate 6	A wash plate (no. 3) containing 400 µL/well of 75 % acetonitrile in PBS/0.03 % CHAPS.
Plate 7	An elution plate containing 75 µL/well of 0.1 % formic acid.

RapidFire/MS and LC/MS Analysis

MRM transitions and collision energies for each surrogate peptide were determined as previously described.[7] The predetermined optimum MRM transitions for antibody-enriched peptide were analyzed on an Agilent 6490 Triple Quadrupole Mass Spectrometer (fitted with a Jet Stream ionization source and running in positive MRM mode) coupled to either an Agilent 1290 Infinity Series LC or an Agilent RapidFire 300 High-throughput Mass Spectrometry System.

The RapidFire/MS platform is a fully automated, online, microfluidic sample preparation system. Samples are aspirated directly from 96-well assay plates and loaded onto a micro-scale solid-phase extraction cartridge. The salts, buffers, and other contaminants are washed through the cartridge while the analytes of interest are retained. The purified analytes are then eluted from the cartridge directly onto the mass spectrometer for analysis as follows (See Tables 2 and 3).

Table 2. LC and RapidFire conditions.

	LC/MS	RapidFire/MS
Column/Cartridge	Agilent ZORBAX 300 SB-C18, 2.1 × 50 mm	A (C4 packing material)
Column temperature	35 °C	Room Temperature
Flow rate	1.2 mL/min	1.25 mL/min
Mobile phase A	0.1 % formic acid in ultrapure water	0.1 % formic acid in ultrapure water
Mobile phase B	0.1 % formic acid in 90 % ultrapure acetonitrile	0.1 % formic acid in 90 % ultrapure acetonitrile
Gradient	10 % – 16 % B in 1 min, to 22 % B at 1.5 min, 40 % B at 1.85 min, 70 % B at 1.9 min, then back to 10 % B from 1.95 min to 3 min	None, wash of 3,000 ms, elution of 3,000 ms, re-equilibration of 500 ms
Total cycle time	5.5 min/sample	~10 s/sample
Injection volume	20 µL	10 µL

Results and Discussion

To assess the value of this approach for general biomarker validation, we used a variety of peptides derived from protein targets differing widely in concentrations in human plasma. Linearity and reproducibility of peptide responses in the RapidFire/MS system was assessed using a range of concentrations of five pairs of pure synthetic tryptic peptides (unlabeled = light, L; stable isotope labeled = heavy, H) dissolved in 0.1 % formic acid. The light and heavy peptides were combined in varying L/H ratios as previously described.[6] The RapidFire system is an SPE-based method, thus all molecules in each sample are injected as a single peak approximately 2 seconds wide (Figure 2). The RapidFire system was able to monitor 10 analyte peptides (5 L + 5 H) that eluted together (Figure 2).

Table 3. MS conditions.

Drying gas	11 L/min, 200 °C
Sheath gas	11 L/min, 250 °C
Nebulizer	30 psig
Nozzle	0 V
Cell acc	4 V
Dwell time	20 ms
Delta EMV	100 V
Resolution	Q1 wide, Q3 unit

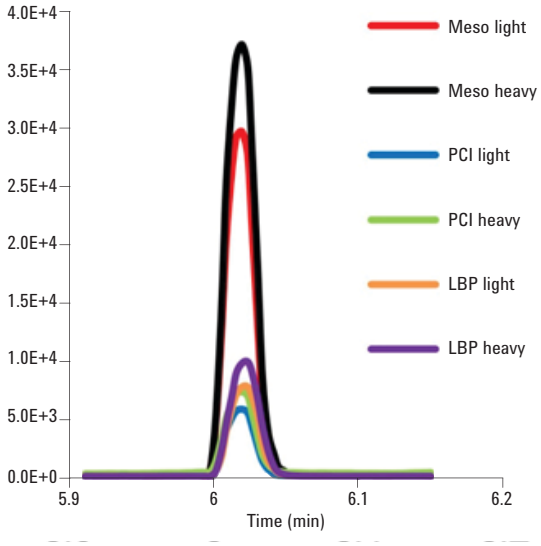

Figure 2. The RapidFire system was able to monitor 10 analyte peptides that eluted together in a peak approximately 2 seconds wide. Six are shown for clarity.

The results of these peptide analyses are shown in Figure 3 and include an example of the RapidFire chromatogram for the mesothelin surrogate peptide. Instrument reproducibility was tested using triplicate samples that formed combination curves (L/H ratio curves) for the five peptide analytes. By pairing unlabeled peptides with stable isotope-labeled, chemically identical versions and measuring varying L/H ratios (a normal procedure in SISCAPA assays) the R^2 values achieved were > 0.99 for four of the peptides and > 0.97 for the surrogate peptide from LPS Binding Protein (LBP). These results suggest that the RapidFire/MS platform can be used for peptide quantitation over at least a 2,500-fold range in abundance. The average percent coefficient of variation (% CV) within-run over the 2,500-fold dynamic range for the five peptides ranged from 6.0 % – 8.3 %. The 10 peptides were combined for this analysis, clearly demonstrating that multiplexed measurement is possible.

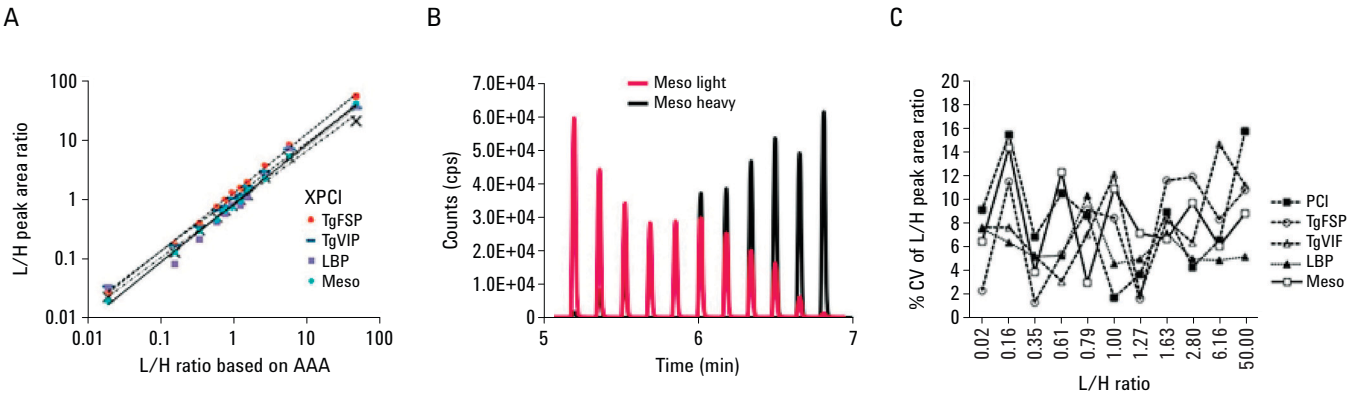

Figure 3. Quantitative plasma peptide analysis using the RapidFire/MS platform. L/H peak area ratios for LPS binding protein (A), mesothelin surrogate peptide spectrum (B) and percent coefficient of variation (% CV) for the five peptides (C).

To demonstrate that the SISCAPA-RapidFire/MS system can be used for measuring analytes in trypsin-digested human plasma, we analyzed replicate SISCAPA peptide response curves for a low-abundance protein, mesothelin at < 10 ng/mL (a level typically observed by SISCAPA-MRM analysis using LC/MS/MS). We compared the results achieved using an Agilent RapidFire 300 coupled to an Agilent 6490 Triple Quadrupole Mass Spectrometer with those gathered using a standard flow Agilent 6490 Triple Quadrupole LC/MS System. Unfractionated, trypsin-digested human plasma was used in all experiments.

The quantity measured for an aliquot of the same eluant of mesothelin was identical between the two MS platforms, as shown in Figure 4. Forward (red) and reverse (black) curves of trypsin-digested human plasma (no SPE cleanup) were used to measure the levels of mesothelin by SISCAPA-RapidFire/MS. The forward curve was generated by titrating the light peptide from 1,000 fmol to 1 fmol, with the last point containing a 0 fmol spike (that is, endogenous peptide level), while the heavy peptide remained constant at 500 fmol. The reverse curve was generated by titrating the heavy peptide from 1,000 fmol to 0.5 fmol while the light spike was kept constant at 500 fmol.

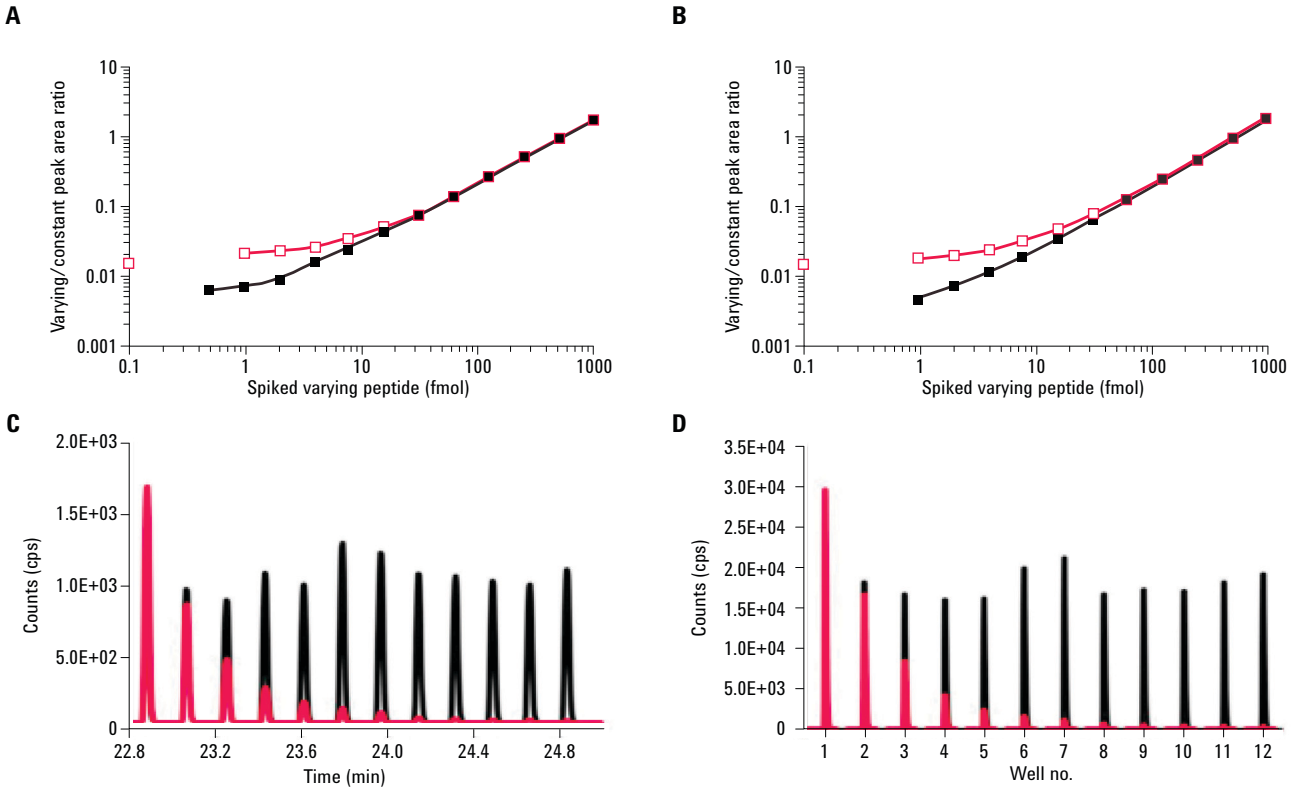

Figure 4. Comparison of analysis of SISCAPA on LC/MS to RapidFire/MS. Analysis of mesothelin using SISCAPA-RapidFire/MS (A); standard flow LC/Agilent 6490 Series Triple Quadrupole LC/MS System (B); MS chromatogram of light (red) and heavy (black) transitions of the mesothelin peptide for the forward curve on the RapidFire/MS (C) and LC/MS platforms (D).

Conclusions

Using an optimized SISCAPA procedure, we were able to generate eluted peptides with sufficient purity to be analyzed by the RapidFire platform without chromatography upstream of MS, thereby decreasing the cycle time to approximately 10 seconds. In this mode, a plate of 96 samples can be analyzed in about 15 minutes and a 1,500 sample validation study in approximately 4 hours. By coupling RapidFire technology with the 6490 Mass Spectrometer, multiplexed analysis of five peptides was achieved (a total of 10 transitions; 5 L and 5 H), representative for multiplexed measurement of biomarkers. We were able to accurately quantify surrogate peptides from mesothelin and LPS binding protein in digested human plasma using the RapidFire/MS platform witha CV of less than 8 %, which is within the range of instrument variation observed when pure peptides are injected. Therefore, incorporation of the RapidFire 300 system coupled to the 6490 Triple Quadrupole Mass Spectrometer in the laboratory enables a high throughput, multiplexed biomarker validation platform.

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