Dynamic MRM Measurements of Multi-Biomarker Proteins by Triple-Quadrupole Mass Spectrometry with Nanoflow HPLC-Microfluidics Chip

Eun Sun Ji[a,b], Mi Hee Cheon[a,c], Ju Yeon Lee[a,d], Jong Shin Yoo[a,c], Hyun-Jin Jung[e], and Jin Young Kim[a]*

[a]*Division of Mass Spectrometry, Korea Basic Science Institute, Yangcheong-ri, Ochang-eup, Cheongwon-gun, Chungbuk, 363-883, Korea*
[b]*Department of Chemistry, Hannam University, Daejeon, 305-811, Korea*
[c]*Graduate School of Science and Technology, Chungnam National University, Daejon, 305-333, Korea*
[d]*Department of Chemistry, Yonsei University, Seoul, 120-749, Korea*
[e]*Agilent technologies Korea Ltd., 906-10, Lui-dong, Yeongtong-gu, Suwon-si, Gyeonggi, 443-766, Korea*

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Abstract: The development of clinical biomarkers involves discovery, verification, and validation. Recently, multiple reaction monitoring (MRM) coupled with stable isotope dilution mass spectrometry (IDMS) has shown considerable promise for the direct quantification of proteins in clinical samples. In particular, multiple biomarkers have been tracked in a single experiment using MRM-based MS approaches combined with liquid chromatography. We report here a highly reproducible, quantitative, and dynamic MRM system for validating multi-biomarker proteins using Nanoflow HPLC-Microfluidics Chip/Triple-Quadrupole MS. In this system, transitions were acquired only during the retention window of each eluting peptide. Transitions with the highest MRM-MS intensities for the five target peptides from colon cancer biomarker candidates were automatically selected using Optimizer software. Relative to the corresponding non-dynamic system, the dynamic MRM provided significantly improved coefficients of variation in experiments with large numbers of transitions. Linear responses were obtained with concentrations ranging from fmol to pmol for five target peptides.

Key words: Dynamic MRM, HPLC-Microfluidics Chip, Protein quantitation, Reproducibility, Biomarker validation

Introduction

In recent years, biomarkers have attracted considerable attention for use in disease diagnosis, prognosis, and therapeutic response monitoring. However, these applications require proper verification and validation in treating the disease of interest.[1] Although ELISA remains the gold standard for clinical applications, liquid chromatography (LC)/multiple reaction monitoring (MRM)-mass spectrometry (MS) methods that utilize stable isotope-labeled internal standards have shown major progress toward the quantification of biomarker proteins.[2,3] These approaches are targeted MS techniques, in which the researcher optimizes the assay for the detection and quantification of specific peptides that are representative of the proteins of interest. MRM-MS has been suggested as an alternative to antibody-based biomarker verification due to its high throughput, selectivity, and sensitivity. Nicol *et al*.[4] employed an immunoaffinity-MRM approach to detect carcinoembryonic antigen (CEA) in sera of patients with lung cancer at nanogram per milliliter levels. Their method correlated strongly with the corresponding ELISA and approached a similar sensitivity.

*Reprint requests to Dr. Jin Young Kim
E-mail: jinyoung@kbsi.re.kr

In MRM analyses, optimizing the selected MRM precursor (Q1) and fragment ion (Q3) pairs for each target peptide is important for establishing the most sensitive MRM assay.[5] This requires judicious selection of the precursor ion charge state and fragment ions combined with empirical tuning of MS parameters. Kuzyk *et al*.[6] presented MRM-based, multiplexed quantification of 45 proteins in human plasma, ranging in concentration from sub-micromolar to millimolar, with no affinity depletion or enrichment. For maximum sensitivity and specificity, they empirically determined the optimal instrumental parameters required to generate the most abundant precursor ions and y-type ion fragments.

The considerable selectivity afforded by the combination of a tandem-MS filter and chromatographic retention times allows a triple-quadrupole LC/MS system to quantify proteins in a multiplexed manner. The recent development of time-resolved MRM data acquisition software, including scheduled MRM and dynamic MRM, has greatly expanded the number of MRM measurements able to be carried out during a single LC-MS run without compromising sensitivity by including elution time constraints for the targeted transitions.[7,8] This allows for quantification of large sets of peptides in a single analysis.

The benefits of time-resolved MRM can be further exploited by coupling with a highly reproducible LC system in which

transit ions can be effectively sequestered in a well-defined and narrow time window. The Nanoflow HPLC-microfluidics chip, which incorporates microfluidic chip technology for nanospray LC/MS, provides the highest analytical performance with regard to run-to-run reproducibility with ease of use, resulting in high-precision quantification.[9,10]

The current study describes the use of a HPLC-microfluidics chip combined with triple-quadrupole MS to yield highly reproducible and quantitative dynamic MRM measurements for the validation of multi-biomarker proteins. Transitions for five target peptides were automatically selected using Optimizer, which is software providing the best fragment ions at optimized collision energy values for each transition. The dynamic MRM measurements were compared to non-dynamic MRM measurements in terms of reproducibility.

Experimental Section

Materials and Sample preparation

The light (^{12}C) and heavy (^{13}C) forms of five peptides were synthesized by Anygen Co. (Kwangju, Korea) including purification by HPLC. The stable isotope-label (^{13}C) was incorporated at the valine, alanine or proline position, resulting in a mass shift of +6, or +7 Da, respectively. HPLC grade acetonitrile and formic acid were purchased from Burdick and Jackson (Muskegon, MI, USA) and Sigma (St. Louis, MO, USA), respectively. Stock solutions (10 pmol/μL) of the individual light and heavy peptides were dissolved in an aqueous of 0.1% formic acid. Mixtures containing either the light or the heavy peptides were prepared at 100 fmol/μL if the concentration has not been mentioned.

Instrumentation and MS analysis

The HPLC-microfluidics chip MRM/Triple quandrupole MS analysis was achieved with Agilent 6460 Triple Quad QQQ (Palo Alto, CA, USA) connected with Agilent ChipCube LC-MS interface (Palo Alto, CA, USA) and an Agilent 1200 Series nanopump system (Palo Alto, CA, USA). Each sample of 1 μL was injected via an autosampler. A HPLC-microfluidics chip (Agilent, Palo Alto, CA, USA), featuring a trapping column (40 nL) and separation column (75 μm × 43 mm, 5 μm, Zorbax) containing C18 particles, was used. Mobile phase A consisted of 97% / 3% / 0.1% water/ACN/formic acid, while mobile phase B was composed of 10% / 97% / 0.1% water/ACN/formic acid. The capillary pump flow rate was set at 2 μL/min for loading and washing the samples using 100% mobile phase A. The LC gradient was delivered at 300 nL/min and performed as follows: 3% B at 0 min, 10% B at 0.5 min, 45% B at 7 min, 90% B at 8 min, 3% B at 8.1 min, stop time at 10 min and post time for 5 min.

Electrospray ionization was accomplished by applying a spray voltage of 1750 V. MS instrument settings included a drying gas of 2.5 L/min and gas temperature of 325 °C. To perform the MRM measurement, 135 eV of fragmentor voltage was applied and individually optimized CE voltage according to peptides by Optimizer software (MassHunter optimizer, version B.03, Agilent, Palo Alto, CA, USA) was used. In the dynamic MRM measurement, transitions were acquired during for retention time ±1 min of corresponding peptide.

Data analysis

Data analysis was performed using an Agilent Mass Hunter Quantitative Analysis Software (version B.04, Palo Alto, CA, USA). All HPLC-microfluidics chip MRM/Triple quandrupole MS analysis were performed in triplicate. In this study, an averaged data within CV<20% were included.

Results and Discussion

Optimized selection of MRM transitions for each peptide

We selected five peptides that represent candidate biomarker proteins based on our preliminary results for colon cancer biomarker discovery, which have been under study. These five peptides were devoid of missed cleavage sites and any amino acids that are susceptible to chemical modification, such as cysteine and methionine. Supplementary Table 1 gives the molecular weights of peptides used in this study.

To ensure the most sensitive combination of precursor and fragment ions, the most intense charge state was determined by ramping the fragmentor voltages during the first quadrupole (Q1) scans. Fragmentor voltages affect the efficiency of transfer of the precursor ion into the MS instrument. Fine-tuning these voltages ensures the maximum MRM signal. The average chromatographic peak area (from three replicate analyses) of each doubly and triply charged precursor ion, with the exception of G-P-K, which gave only doubly charged precursor ions, are represented in Supplementary Figure 1. The intensities of most precursor ions increased with fragmentor voltages

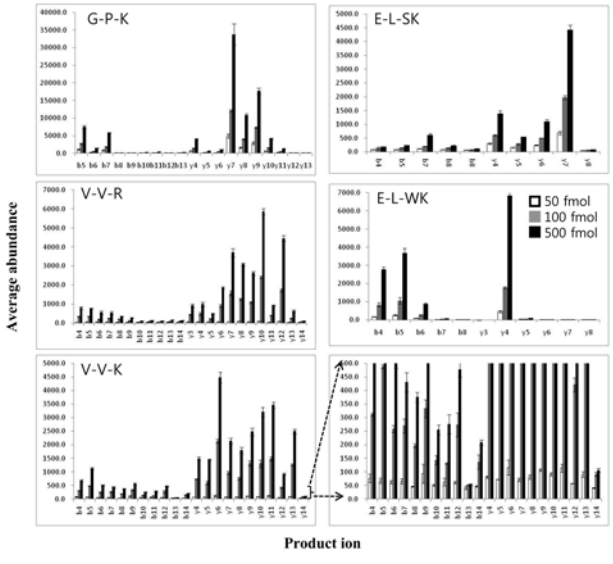

Figure 1. Average abundances of fragment ions for each peptide of interest.

up to 130 eV, reaching saturation between 130 and 140 eV. Since most peptides showed their most abundant precursor ions within this range, 135 eV was selected as the optimum fragmentor voltage. Doubly charged precursor ions for peptides V-V-R, E-L-SK, G-P-K, and V-V-K, and triply charged precursor ions for E-L-WK, were selected in the Q1 scan.

Next, Optimizer software was used to determine which product ions were dominant in the collision-induced dissociation (CID) fragmentation patterns generated from each peptide to create a transition, *i.e.*, a MRM Q1/Q3 ion pair. Each peptide was injected onto a HPLC-microfluidics chip and analyzed using transitions containing all possible b- and y-series fragment ions while ramping the collision energy (CE). This approach resulted in CE voltages that maximized the generation of each fragment ion species. CE voltages were varied by ± 4 V relative to the equation-derived value (CE = 0.036**m/z* – 4.8, suggested by Agilent) for up to eight steps.

Figure 1 presents the average highest abundance of each CID fragment ion obtained at individually optimized CE voltages. Fragment ions were each generated differently due to different peptide residues and proton mobility. However, y-type ions tended to be dominant. Although Optimizer experiments were performed with peptides at concentrations of 50, 100, and 500 fmol, each experiment resulted in the same order of abundance of fragment ions. Note, however, that the V-V-K peptide at 50 fmol yielded fragment ions with low intensity (under 100 in peak area) and its fragmentation pattern differed from those obtained at 100 and 500 fmol. These low intensities suggest that the selected transition was not appropriate for quantitative studies.[2] The relationship between the coefficients of variation (CVs) and the peak area of each transition was examined and is shown in Supplementary Figure 2 for all Optimizer experiments. The figure indicates that peak area values under 250 are unlikely to yield acceptable CVs (<20%).

Dynamic MRM for multiplexed peptide detection

A physical limit exists to the number of transitions that can be monitored reliably during a single LC/MRM-MS run. Thus, the number of peptides that can be detected and quantified precisely is also limited. This limit arises from the fact that the duty cycle is directly proportional to the number of transitions included in the experiment. Consequently, a trade-off exists between the number of transitions and the limit of detection. A large number of transitions results in insufficient sampling of data points across the chromatographic elution profile of a peptide and inaccurate quantification. And, a reduction in dwell time reduces the signal-to-noise ratio and correspondingly increases the limit of detection.[7]

To increase the total number of transitions that can be measured in a single LC/MRM-MS experiment without appreciable loss of sensitivity, a time constraint was added. This time constraint is manifest in a dynamic algorithm (dynamic MRM measurement) that allows the system to acquire transitions only during the retention window of each eluting peptide.[8] In this study, the acquisition time for a given transition was restricted to the retention time ± 1 min with individually optimized instrument parameters as described above.

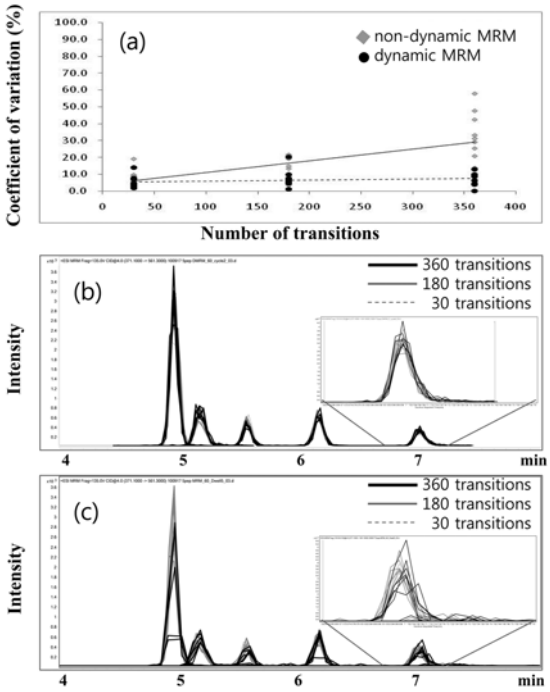

Figure 2. Comparisons of dynamic and non-dynamic MRM measurements. (a) The CVs are shown as a function of transition; (b) and (c) Overlaid base peak ion chromatograms (18 total) obtained from dynamic and non-dynamic MRM measurements, respectively.

Figure 2a shows the relationship between the CV and the number of transitions in which 30, 180, and 360 transitions were measured with and without time constraints. In measurements without time constraints (non-dynamic MRM), whereby each transition was monitored during the entire LC/MS run, CVs increased with the number of transitions. Large numbers of transitions resulted in shorter dwell times during a given cycle (about 2 s for this study), thereby resulting in lower signal quality and higher CVs. Dynamic MRM measurements, in contrast, segmented the total number of transitions in accordance with their elution from the LC. Long dwell times resulted in increased signal-to-noise ratios and the small number of concurrent transitions improved selectivity as the nonspecific biochemical background was reduced.

Figures 2b and c present overlaid base peak ion chromatograms obtained from dynamic and non-dynamic MRM measurements including 30, 180, and 360 transitions, respectively. Eighteen base peak ion chromatograms from three replicate analyses of ten target peptides (five natural peptides and five isotope-labeled peptides) are overlaid. The benefits of dynamic MRM measurements in quantitative analyses are clear. All dynamic MRM measurements provided reproducible peak area regardless of the number of transitions. However, in non-dynamic MRM measurements, peak area was greatly affected by the number of transitions.

In terms of retention time reproducibility and resolution, the HPLC-microfluidics chip exhibited excellent performance with CVs of less than 1% between each 30 min gradient run (Figure 2b). The average elution window was 6–8 s at half the peak height. Since longer dwell times resulted in higher sensitivity, the cycle time was increased from 2 to 4 s. The results from these two experiments are compared in Figure 3. Note that the CVs of the transitions increased despite the twofold increase in dwell time from 10–163 ms to 23–329 ms for each transition (Figure 3a). In dynamic MRM measurements, dwell time was automatically determined according to the number of concurrent transitions under a given cycle time. A cycle time of 4 s was too long to obtain the minimum number of data points required to quantify peptides eluted from the HPLC-microfluidics chip within about 15 s (Figure 3c). To enhance signal quality, the cycle time was generally set to obtain at least six points across the elution peak.[5,7] Therefore, an appropriate cycle time, based on the width of the chromatographic peak, should be considered in LC/MRM-MS experiments.

Response curves for dynamic MRM measurements of five target peptides

To determine the linear response of dynamic MRM measurements, five target peptides were spiked into a blank solution with five stable isotope-labeled peptides. Four of the five target natural peptides were diluted from 0.3 fmol/μL to 3000 fmol/μL and G-P-K target natural peptides were diluted from 0.6 fmol/μL to 6000 fmol/μL. Five stable-isotope labeled peptides were diluted to the same concentration of 300 fmol/μL. The experi- mental peak area ratios of the natural to heavy peptides are plotted in Supplementary Figure 3. A linear response was observed over three orders of magnitude in peptide concentration. The detection limit (CV<20%) was calculated as 6 fmol/μL for G-P-K, 30 fmol/μL for EH-WK, and 3 fmol/μL for the other peptides.

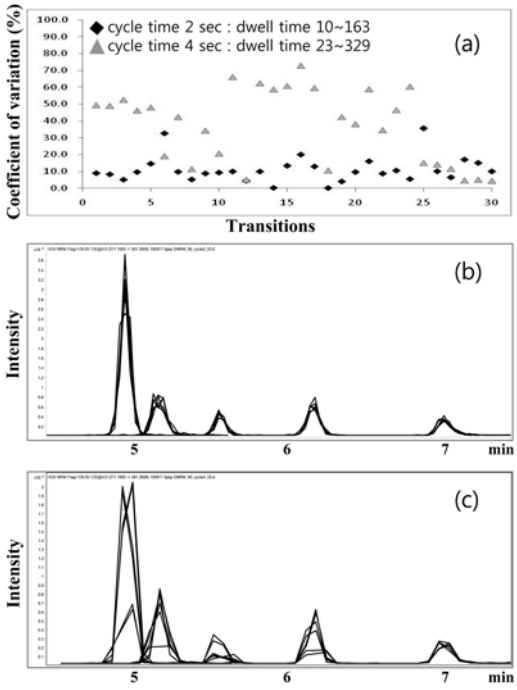

Figure 3. Comparisons of cycle time in dynamic MRM measurements using HPLC-microfluidics chip/Triple-Quadrupole MS: (a) CVs of the peak area; (b) and (c) overlaid base peak ion chromatograms (6 total) obtained from dynamic MRM measurements at cycle time 2 and 4 s, respectively.

Conclusions

The performance of dynamic MRM measurements was demonstrated for highly reproducible and quantitative validation of multi-biomarker proteins using a Nanoflow HPLC-microfluidics chip/Triple-Quadrupole MS. This technique enabled reproducible measurements of a large number of analytes to be performed during the course of a single LC/MS experiment without compromising sensitivity. Transitions were selected by Optimizer software. CVs and the signal quality of chromatograms obtained from dynamic MRM measurements were significantly improved relative to those of non-dynamic systems. A linear response was observed for peptide concentrations ranging from 3 fmol to 3000 fmol. This method can be used to expand the scope of LC/MRM-MS-based clinical applications.

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