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Ultrasensitive Sample Quantitation via Selected Reaction Monitoring Using CITP/CZE-ESI-Triple Quadrupole MS

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Abstract

We demonstrate the direct coupling of transient capillary isotachopheresis/ capillary zone electrophoresis (CITP/CZE) with a high sensitivity triple quadrupole mass spectrometer operating in selected reaction monitoring (SRM) mode for sample quantitation. The capability of CITP/CZE for *in situ* sample enrichment and separation has been shown to significantly improve the analytical figures of merit. A linear dynamic range spanning 4 orders of magnitude was observed. An average signal-to-noise ratio (S/N) of 49.6 was observed for 50 attomoles of targeted peptide in the presence of a complex and much more abundant bovine serum albumin (BSA) digest product. A correlation of variation (CV) less than 10 % for peak area was measured from triplicate sample analyses at 50 pM peptide concentration, showing good reproducibility of this online CITP/CZE-SRM mass spectrometry (MS) platform, and with limit of quantitation (LOQ) demonstrated to be well below 50 pM.

Introduction

Capillary electrophoresis (CE) coupled with mass spectrometry (MS) has been well recognized as a complementary analytical technology to more conventional liquid chromatography-mass spectrometry (LC-MS). By combining the high resolving power of CE with the highly sensitive and information-rich detection of MS, this hyphenated technique has enjoyed successful applications in many fields of scientific research, such as biomarker discovery and verification^{1–4}, metabolomics^{5–6}, identification and quantification of environmental pollutants⁷ and food quality control⁸. While much less widely applied than LC-MS, the number of publications related to development and application of CE-MS continues to increase due to its potential capability in performing sensitive and high throughput sample analysis^{9–11}. Online coupling of CE with electrospray ionization (ESI)-MS has been demonstrated via a co-axial sheath-flow interface¹² or a sheathless interface using either a metal sheath capillary¹³, a metal coated capillary¹⁴ or a T-junction design that connects separation capillary with electrospray tip¹⁵. With the goal to refine this technology and broaden its utility, every aspect of the CE-MS technique has attracted attention, including CE capillary modification^{16–18}, ESI emitter fabrication¹⁹ and high efficiency CE-MS interface design²⁰. Recently, Maxwell and co-workers have designed a stainless hollow needle with a beveled spray tip as a more robust sheath liquid interface²¹. A conductive tip made by HF etching a fused silica capillary to porous was introduced by Moini²². The performance of sheath-less setup utilizing the etched porous tip was demonstrated by Busnel *et al.*²³, showing its capability in performing CE-ESI-MS analysis in a broad range of flow rates (from low nL/min to sub μ L/min) with exquisite sensitivity. Two CE operation modes, capillary zone electrophoresis (CZE) and transient capillary isotachopheresis/capillary zone

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electrophoresis (CITP/CZE), have shown to be most compatible with online MS analysis and have thus been most widely used. Heemskerk *et al.* reported an improved ionization efficiency for phosphopeptides using CZE-ESI-MS under ultra low ESI flow rate (below 20 nL/min) recently²⁴. Ramautar and co-workers reported enhanced coverage of urinary metabolome using CITP/CZE-ESI-MS²⁵. Busnel and co-workers applied both CZE and CITP/CZE in a sheathless CE-ESI-MS setup and reported nanomolar and subnanomolar detection limits respectively²³. Thus far, CE-MS has been primarily used for the qualitative analysis of biomolecules or small molecules. With the growing interest in identifying biomarkers for diagnostic, prognostic and predictive applications, new analytical tools capable of effectively quantifying low abundant targets in complex biological matrix are needed.

Triple quadrupole MS operating in a selected reaction monitoring (SRM) mode has demonstrated extremely high sensitivity, dynamic range and specificity in quantifying targeted analytes in a complex chemical or biological sample matrix²⁶. The SRM MS-based quantitation method has been extensively investigated and primarily used in combination with LC for quantitation of metabolites^{27–28}, and increasingly for peptide measurements, e.g. verification of protein biomarkers^{26,29–30}. Online coupling CE with SRM MS, though still in its infancy, has already demonstrated its unique capability in sample quantitation with significantly less sample consumption as compared to LC-SRM MS based technique. Jeong *et al.* recently demonstrated the detection and quantitation of underivatized amino acids using CZE-SRM MS via a sheathless interface³¹. Li and co-workers have reported a highly sensitive method using CZE-SRM MS³², and 10- to 20-fold sensitivity improvements over HPLC-SRM MS were demonstrated as well as linear response for Leu-enkephalin from 1.5 nM to 10 μ M in a BSA tryptic digest background.

Here we report the use of CITP/CZE for online SRM MS quantitation. CITP is a static-state electrophoresis, which utilizes the conductivity difference between sample ions and background ions to achieve a sample stacking mechanism. CITP has been mainly used as a preconcentration method prior to sample separation by which a concentration factor of ~1000 can be achieved when applied to dilute samples. CITP/CZE is a hybrid mode capable of achieving both sample concentration (selectively) and separation simultaneously³³. As a result of the selective focusing and high resolving power, trace compounds are less masked by the major compounds after CITP/CZE and thus become more detectable by MS. Fang *et al.* have demonstrated the use of CITP/CZE MS for candidate biomarker discovery using tissue samples^{34–35}. Our goal is to utilize the intrinsic focusing feature of CITP/CZE to achieve ultrasensitive sample quantitation and evaluate the lower limit of quantitation using CITP/CZE-SRM MS for quantifying targeted peptides in complex biological matrix.

EXPERIMENTAL

Chemicals

Bovine serum albumin (BSA) tryptic digest standard was purchased from Protea (Morgantown, WV). Leu-enkephalin, angiotensin II, kemptide, ammonium acetate, methanol and acetic acid were purchased from Sigma (St. Louis, MO). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ).

Sample preparation

BSA tryptic digest solution was used to evaluate the performance of the online CE-ESI-MS platform. Stock solution of BSA digest was prepared by dissolving 500 pmol of BSA digest standard in 100 μ L leading electrolyte (LE) solution consisting of 25 mM ammonium acetate in deionized water with the solution pH adjusted by adding acetic acid to pH=4. Working solutions with concentrations ranging from 10 nM to 3.75 μ M were obtained by

diluting stock solution with LE. Preparation of samples for CZE experiments was similar except LE was substituted with 0.1 M acetic acid in deionized water. 0.1 M acetic acid solution was subsequently mixed with methanol at volume ratio 9:1 as background electrolyte (BGE) solution for better electrospray operation.

A mixture of three peptides (Leu-Enkephalin, Angiotensin II and Kemptide) spiked into BSA tryptic digest at different concentrations were used in the CITP/CZE-SRM MS sample quantitation experiments. Stock solution with 10 μ M concentration for each peptide was prepared in water and stocked in aliquots. The stock solution was then diluted using BSA background matrix (50 nM BSA digest in 25 mM LE) to obtain a peptide concentration series for CITP/CZE-SRM MS quantitation.

CITP/CZE-ESI-MS interface

Figure 1 shows the schematic of the interface developed to couple CITP/CZE online with an ESI-triple quadrupole (QQQ) mass spectrometer. The CITP/CZE separation capillary (75 μ m i.d., 150 μ m o.d., and 88 cm long) was inserted into a large bore capillary (200 μ m i.d., 360 μ m o.d., and 5 cm long) concentrically using a PEEK cross. The free end of the large bore capillary was first pulled into a fine tip using a Sutter P-2000 laser puller and then chemically etched to remove a part of the tip using HF solution¹⁹ to form an ESI emitter with a $\sim$ 50 μ m opening, as shown by the inserted picture at the bottom right of the Figure 1. The tapered i.d. of the large bore ESI emitter capillary also allowed the separation capillary to be inserted as far as possible until it hit the inner wall of the emitter capillary to both minimize the dead volume and provide a consistent coupling between the CITP/CZE separation capillary and the ESI emitter, as shown by the inserted picture at the bottom middle of the Figure 1. Before each CITP/CZE-ESI-QQQ MS analysis, a sheath flow of BGE serving as electrically conductive solution was introduced to completely fill the annular space between the separation capillary and the ESI emitter capillary. The liquid junction formed at the ESI emitter allows a second voltage to be applied for stable electrospray operation and maintains the electric contact. To minimize the sample dilution, the sheath-flow was turned off during the CITP/CZE-ESI-QQQ MS analysis. To avoid electric contact interruption by the air bubbles generated from the redox reaction at the metal contact of the ESI voltage, which could rapidly damage the separation capillary due to the high separation voltage ($\sim$ 30 kV), the second voltage for ESI was applied to the sheath flow waste line through a flow split design, as shown in Figure 1, which was flushed with the sheath liquid to avoid bubble accumulation after each experiment.

The separation capillary was coated with hydroxypropyl cellulose to minimize electroosmotic flow. The separation capillary was filled with BGE solution before each experiment. Samples were loaded into the separation capillary by the hydrodynamic method³⁶ at constant gas pressure (1 psi). Sample loading was adjusted by varying the length of loading time. The CITP/CZE separation voltage of 27 kV was applied to the sample inlet of the capillary using a Glassman high voltage power supply. A $\sim$ 1 kV electric potential at the ESI emitter liquid junction was created under the separation voltage when the sheath flow liquid waste line was grounded. Analytes were eluted from the separation capillary hydrodynamically by applying 1 psi nitrogen back pressure to the trailing electrolyte (TE) reservoir (Figure 1) after a fixed separation time, determined experimentally for achieving optimum ion focusing and separation.

MS optimization and data processing

An Agilent QQQ mass spectrometer (model 6430) was used for all sample analyses. The standard ESI interface of the instrument was modified with a multi-capillary/dual ion funnel interface³⁷ making the performance of the instrument similar to Agilent's latest triple

quadrupole MS product (model 6490), as shown by the inserted picture at the bottom left of the Figure 1. The ESI voltage of the MS was varied in the range of -200 to -500 V for the optimum ESI sensitivity and signal stability considering the ~ 1 kV potential at ESI emitter liquid junction as described above under CITP/CZE operating condition. A dry nitrogen flow of 10 L/min and a gas temperature of 320°C were used for optimum MS sensitivity. Both full scan MS and SRM were used in this study. In SRM mode, specific mass to charge ratio (m/z) precursor ions were selected in the first quadrupole (Q1) and fragmented in the second quadrupole (Q2 collision cell) via collision induced dissociation under sufficient collision energy. The specific m/z product ions were then selected in the third quadrupole (Q3) and monitored by the MS detector. Each selected precursor/product ion pair in SRM MS is referred to as a SRM transition. Through monitoring the unique transitions of the selected analytes, both high sensitivity and specificity can be achieved in sample quantification. SRM MS operating parameters for each peptide in this study were optimized using direct infusion of the peptide standard with $1\text{ }\mu\text{M}$ concentration at 500 nL/min . Three most intense fragments were selected for each peptide as SRM transitions regardless of the fragment ion types. The collision energy for each SRM transition was optimized and recorded for the highest product ion intensity. The dwell time for each SRM transition was set at 65 milliseconds to ensure at least 12 data points for each analyte peak in extracted CITP/CZE SRM MS chromatogram. All the SRM transitions and their related optimum collision energies for three peptides selected for SRM MS quantitation in this study are listed in Table 1.

Data analysis was performed by using Agilent MassHunter Qualitative Analysis Software (version B.02.00). Symmetric $0.5\text{ }m/z$ tolerance was used to extract the selected ion chromatograms. Unit resolutions were used for both Q1 and Q3 in SRM data acquisition. Peak retention time, peak area and peak width were obtained through manual integration. In S/N calculation, peak height was used as signal and the software default setting, peak-to-peak definition, in the range of 3-minutes before and after the target peak was used for noise measurement. In this default setting, the noise value for the selected range is calculated as the difference between the highest and the lowest peak abundance values.

RESULTS AND DISCUSSION

CITP/CZE sample loading capability and optimization

As one of the unique features, CITP is capable of selectively enriching/focusing the low abundance compounds in a complex mixture. During the CITP stacking process, analytes migrate into different bands under a constant electric field according to their different electric mobility. At the completion of the CITP stacking process, low abundance analytes are concentrated to a much greater extent than the major components in a mixture³³. The CITP stacking mechanism enables relatively large ($\sim\mu\text{L}$) sample loading as compared to the much smaller ($\sim\text{nL}$) sample loading of traditional CZE, providing a basis for more sensitive detection for trace analytes. The CITP/CZE separation is a hybrid technique capable of simultaneously achieving high sensitivity and resolution by a combination of ion stacking and separation processes. To evaluate capability of CITP/CZE relative to CZE, both CITP/CZE and CZE were coupled online with ESI-MS. Figure 2 compares the analyte intensity between CITP/CZE-ESI-MS and CZE-ESI-MS at two different sample loading time using BSA tryptic digest. With 1.5 min sample loading time, which yielded a small sample plug, equivalent ion intensity was measured between CITP/CZE-ESI-MS and CZE-ESI-MS, as shown by the base peak ion chromatograms (BPC) in Figure 2A (the top two panels) and the extracted ion chromatograms (EIC) for selected peptide ($m/z = 432.3$) in Figure 2B (the top two panels). Similar peak widths (0.456 min and 0.499 min) were also observed between CITP/CZE-ESI-MS and CZE-ESI-MS at this short sample loading time. It is also expected that different peptide peaks will be more resolved in CZE-ESI-MS as compared to CITP/

CZE-ESI-MS, as shown in Figure 2A, due to the additional time required to complete the CITP ion stacking process before the analyte peaks are separated from each other in the CITP/CZE-ESI-MS. When the sample loading time was increased to 3 min, corresponding to an injection of a longer sample plug, the benefit of CITP/CZE-ESI-MS becomes more obvious, as shown by the bottom two panels of Figures 2A and 2B. While CZE-ESI-MS yielded essentially no gain in peak intensity and showed peak broadening (0.711 min), the analyte peak intensity in CITP/CZE-ESI-MS was increased by approximately 4-fold while the peak width remained essentially the same (0.477 min). The increased sample loading volume in CITP/CZE-ESI-MS also resulted in the detection of more analyte peaks due to the effective ion focusing. The apparent nonlinear dependence between sample loading time and the detected analyte peak intensity under these two loading time in CITP/CZE-ESI-MS is due to the hydrodynamic sample loading method used in the experiment. Under the small gas pressure (~1 psi), which was kept at constant by using a high precision digital gas pressure regulator, there is an approximately one minute time delay between the application of the gas pressure and fully developed liquid flow in the separation capillary. With this considered the detected peak intensity in CITP/CZE-ESI-MS is proportional to the sample loading time (i.e. the size of the sample plug).

In order to optimize the sample loading capacity of CITP/CZE for the length of the separation capillary and the separation voltage used in the experiment, a series of different sample loading times ranging from 1.5 minutes to 15 minutes were evaluated and the ion intensity was measured at each sample loading time using BSA tryptic digest. Figure 3A shows the BPCs from the CITP/CZE-ESI-MS measurements at different sample loading time. The optimum loading time under the current CITP/CZE setup is defined as the maximum sample loading volume to allow the sample stacking and separation to be fully developed in the remaining length of the capillary. As indicated by the experimental measurements in Figure 3A, peptide intensity continues to increase as the sample loading time increases from 1.5 minutes to 7.5 minutes while the electrophoresis peak width remains essentially the same. Beyond 10 minutes sample loading time, significant peak broadening and loss of separation quality was observed. This observation was further implied by the AUC (area under curve) of BPC vs. loading time in Figure 3B. As sample loading was increased beyond 10 minutes, the rate of area increase (e.g. the slope of the curve) was decreased. This is largely due to the peak overlapping as a result of insufficient separation time at large sample loading time. Consequently, 7.5 minutes sample loading time was selected as the optimum sample loading time, corresponding to about 1 μ L of sample loading volume or 25% of the total separation capillary volume, since it provided the best balance among sample loading volume, completion of sample stacking and sufficient analyte separation. The variation of the retention time (RT) under different sample loading time, as shown in Figure 3C for BSA peptide m/z 432.3 [VGTR+H]⁺, is due to a combination of ion focusing time and separation time change in CITP/CZE operation mode. In the pure CITP mode, the ion focusing time is expected to increase as the sample loading time increases. In the pure CZE mode, the sample retention time decreases as the sample loading time increases. The final RT in CITP/CZE mode is determined by both ion focusing time and separation time. The data in Figure 3C indicate that RT increases initially as sample loading time increases due to the dominant change of ion focusing time. Beyond 7.5 min sample loading time, RT decreases as the sample loading time increases indicating the change of ion separation time becomes the dominant factor in determining the change of sample RT. Good reproducibility of RT was also measured with the variance smaller than 4%.

CITP/CZE-ESI-MS sample quantitation

The initial evaluation of CITP/CZE-ESI-MS for sample quantitation was performed by using BSA digest solution of different concentrations ranging from 20 nM to 3.75 μ M. The peak areas for randomly selected BSA peptides were measured from triplicate CITP/CZE-ESI-MS analyses of each sample concentrations. Full scan MS from m/z 300 to 2250 was used in the experiments. Figure 4 shows the peak areas for two selected peptide ions m/z 582.5 [LVNELTEFAK+2H]²⁺ and m/z 432.3 [VGTR+H]⁺ at different BSA tryptic digest solution concentrations. Good reproducibility (CV<24%) and linearity were observed for both peptides in the tested BSA concentration range.

To further determine the limit of quantitation (LOQ) for the CITP/CZE-ESI-MS instrument platform and investigate the matrix effect on the instrument LOQ, SRM MS analysis, as the most sensitive and specific MS operating mode, was used to quantify a set of samples containing three peptides (Leu-enkephalin, Angiotensin II and Kemptide) spiked into 50 nM BSA tryptic digest solution at different concentrations ranging from 50 pM to 5 μ M. With an estimated 1 μ L sample loading volume, the corresponding peptide loading amount was from 50 attomoles to 5 picomoles. Each sample solution also contains 25 mM LE as required by CITP/CZE separation. Three SRM transitions were monitored for each spiked in peptide and triplicate CITP/CZE-ESI-MS analyses were performed at each sample concentration. One additional SRM transition from a BSA peptide (m/z 432.3 [VGTR+H]⁺) was also monitored during each CITP/CZE-ESI-MS analysis to ensure the stability of the instrument. This additional transition can also be used to normalize the peak intensity of the targeted peptides improving the precision of the quantification.

Different ion focusing was initially observed for three different spiked in peptides during the CITP/CZE-ESI-MS analyses under the current CITP/CZE-ESI-MS setup and operating conditions. Figure 5 shows the selected ion chromatograms for these peptides corresponding to SRM transitions 556.3 to 397.3 for Leu-enkephalin, 523.8 to 784.5 for Angiotensin II, and 386.7 to 567.4 for Kemptide respectively. Leu-enkephalin peak in Figure 5 is shown to be much broader than Kemptide and Angiotensin II peaks along with lower intensity indicating a poor CITP focusing for Leu-enkephalin ions. This different ion focusing characteristic can be explained by the Kohlrausch equation³⁸,

$$C_A = C_L / \{ [\mu_L / (\mu_L + \mu_R)] [(\mu_A + \mu_R) / \mu_A] \}, \quad (1)$$

Where C_A is the final analyte concentration, C_L is the concentration of LE, μ is electrophoretic mobility with A and R stands for analyte and counter ion respectively. Given constant leading electrolyte concentration, the final concentration of the analyte is determined by its electrophoretic mobility μ_A . The order of electrophoretic mobility for these three peptides is Kemptide > Angiotensin II > Leu-enkephalin based on their relative elution time. This electrophoretic mobility order is consistent with their peak width and peak intensity shown in Figure 5 as analyte with higher electrophoretic mobility gets better focused and thus forms a narrower peak with higher intensity.

Figure 6 shows the CITP/CZE-ESI-SRM MS quantitation curves for Angiotensin II (Figure 6A) and Kemptide (Figure 6B) using the same SRM transitions as Figure 5. Good linearity was observed from 50 pM to 500 nM for Angiotensin II and 50 pM to 100 nM for Kemptide. The EICs for the lowest concentration of each corresponding peptide from three replicates are also shown in the figure with signal-to-noise ratio (S/N) being 63.2, 32.6, 52.9 for Kemptide and 13.5, 21.1, 9.3 for Angiotensin II, respectively. Judging from the S/N, the LOQ for Angiotensin II is about 50 pM while the LOQ for Kemptide is significantly less than 50 pM. The shift of the linear dynamic range to a lower concentration range for

Kemptide may be primarily attributed to the better electrospray ionization efficiency of Kemptide than that of Angiotensin II aside from their focusing ability difference, which is consistent with the larger slope of the trendline for Kemptide (e.g. 4×10^{12} and 2×10^{12} for Kemptide and Angiotensin II respectively), indicating a better CITP/CZE-ESI-SRM MS sensitivity for Kemptide. Due to the relative poor CITP focusing for Leu-enkephalin ions as compared to Kemptide and Angiotensin II ions, as discussed above, the LOQ for Leu-enkephalin was estimated to be about 500 nM (data not shown). Table 2 further lists the CV values for CITP/CZE-ESI-SRM MS measurements at each concentration for both peptides. Median CV values of 12% and 18% for Kemptide and Angiotensin II indicate a good reproducibility for CITP/CZE-ESI-SRM MS sample quantitation. Significantly better LOQ (~ 30 times better) is demonstrated through this study for the CITP/CZE-ESI-SRM MS sample quantitation as compared to a recent study using CZE-ESI-SRM MS³². The extracted ion chromatograms from the triplicate sample analyses shown in Figure 6 also indicate a good reproducibility in peak retention time (RSD 0.8% and 0.9% respectively). The retention time RSD among triplicate sample analyses across the concentration series is smaller than 5% with an average of 2.4% for Angiotensin II and 3.6% for Kemptide. Considering the RT variation introduced by the manual control of the data acquisition in, the RT reproducibility can be further improved by a fully automated data acquisition.

CONCLUSIONS

This study demonstrates the online coupling of CITP/CZE separations with triple quadrupole-based SRM MS by using a high efficiency interface to achieve high sensitivity sample quantitation. A LOQ of less than 50 pM was demonstrated by the CITP/CZE-ESI-SRM MS analysis of a mixture of 3 peptides spiked in a BSA digest sample matrix at different concentrations. A linear dynamic range of 4 orders of magnitude was achieved with excellent reproducibility. The relatively short (30 min) analysis time using CITP/CZE-ESI-SRM MS also makes it suitable for high throughput sample analysis. We also noted the importance of the peptide selection in the optimum CITP/CZE-ESI-SRM MS quantitation due to the different peptide focusing abilities and ionization efficiencies. The selection of peptide SRM transition and optimization of CITP/CZE-ESI-SRM MS operating condition largely dictated the achievable instrument LOQ.

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Figure 2.

Comparison of CITP/CZE and CZE with different sample loading time using BSA digest for selected ion m/z 432.3 [VGTR+H]⁺. (A) Base peak chromatogram (BPC) and (B) Extracted ion chromatograph (EIC). Sample condition: 3.75 μ M BSA digest in 25 mM ammonium acetate (pH=4) for CITP/CZE, 3.75 μ M BSA digest in 0.1 M acetic acid for CZE; Loading was performed using hydrodynamic method under 1 psi pressure.

Figure 3. Sample loading capacity optimization. (A) BPC from CITP/CZE-ESI MS with different sample loading time. (B) AUC (area under the curve) of BPC presented in panel A with different sample loading time. (C) Retention time of peptide m/z 432.3 [VGTR+H]⁺. Sample condition: 3.75 μ M BSA digest in 25 mM ammonium acetate (pH=4), loading condition: constant pressure at 1 psi.

Figure 4.

Peak area calibration curve, for selected BSA peptide ions of m/z 582.5 [LVNELTEFAK +2H]²⁺ (A) and m/z 432.3 [VGTR+H]⁺ (B) at different sample concentrations extracted from CITP/CZE-ESI MS measurements.

Figure 5. EIC for Leu-Enkephalin (top), Angiotensin II (middle) and Kemptide (bottom) at 100 nM concentration spiked in 50 nM BSA digest sample matrix showing different CITP ion focusing characteristics.

Figure 6. Calibration curve of CITP/CZE-ESI-SRM MS quantitation of Angiotensin II (A) and Kemptide (B) and EIC from three replicate SRM measurements at 50 pM sample concentration (right).

Table 1

SRM transitions and parameters used in this study

Compound Name	Sequence	Precursor Ion (m/z)	Product Ion (m/z)	Dwell Time (msec)	Collision Energy (V)
Leu-enkephalin	[YGGFL+H] ⁺	556.3	397.3 (a_4^+)	65	14
		556.3	425.3 (b_4^+)	65	12
		556.3	278.3 (b_3^+)	65	22
Angiotensin II	[DRVYIHPF+2H] ²⁺	523.8	784.5 (b_6^+)	65	14
		523.8	756.5 (a_6^+)	65	20
		523.8	647.4 (b_5^+)	65	20
Kemptide	[LRRASILG+2H] ²⁺	386.7	567.4 (b_5^+ -NH ₃)	65	17
		386.7	549.4 (b_5^+ -NH ₃ -H ₂ O)	65	18
		386.7	539.4 (a_5^+ -NH ₃)	65	22
<i>BSA peptide</i>	<i>[VGTR+H]⁺</i>	<i>432.3</i>	<i>230.1</i> (a_3^+)	<i>65</i>	<i>20</i>

The bolded transitions indicate the transition used for quantitation of each peptide. The non bolded/italicized transitions were used to confirm the peptide's identity. The italicized transition of BSA peptide was used to monitor the platform's reproducibility.

Table 2

CV from triplicate SRM MS analyses of Kemptide and Angiotensin II at each concentration for selected SRM transitions.

Sample Concentration (Mol/L)	Kemptide (386.7–567.4)	Angiotensin II (523.8–784.5)
	Peak area (%CV)	Peak area (%CV)
5.00E-11	9.3	25
1.00E-10	3.0	21
2.50E-10	16	6.1
5.00E-10	2.8	24
1.00E-09	13	29
5.00E-09	16	3.3
1.00E-08	11	15
5.00E-08	12	24
1.00E-07	8.5	16
5.00E-07		4.8