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Short Communication

# Evaluation of multiple reaction monitoring cubed for the analysis of tachykinin related peptides in rat spinal cord using a hybrid triple quadrupole-linear ion trap mass spectrometer

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ABSTRACT

Targeted peptide methods generally use HPLC–MS/MRM approaches. Although dependent on the instrumental resolution, interferences may occur while performing analysis of complex biological matrices. HPLC–MS/MRM$^3$ is a technique, which provides a significantly better selectivity, compared with HPLC–MS/MRM assay. HPLC–MS/MRM$^3$ allows the detection and quantitation by enriching standard MRM with secondary product ions that are generated within the linear ion trap. Substance P (SP) and neurokinin A (NKA) are tachykinin peptides playing a central role in pain transmission. The objective of this study was to verify whether HPLC–MS/MRM$^3$ could provide significant advantages over a more traditional HPLC–MS/MRM assay for the quantification of SP and NKA in rat spinal cord. The results suggest that reconstructed MRM$^3$ chromatograms display significant improvements with the nearly complete elimination of interfering peaks but the sensitivity (i.e. signal-to-noise ratio) was severely reduced. The precision (%CV) observed was between 3.5% and 24.1% using HPLC–MS/MRM and in the range of 4.3–13.1% with HPLC–MS/MRM$^3$, for SP and NKA. The observed accuracy was within 10% of the theoretical concentrations tested. HPLC–MS/MRM$^3$ may improve the assay sensitivity to detect difference between samples by reducing significantly the potential of interferences and therefore reduce instrumental errors.

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## 1. Introduction

Neuropeptides play a central role in pain transmission and their quantification provides valuable insight on the molecular events associated with the pathophysiology. Moreover, neuropeptides can be used as biomarkers to support the development of new analgesic drugs and assist the decision making process in non-clinical drug development [1–3]. Mass spectrometry (MS) has a significant role in bioanalytical chemistry workflow. Adequate method selectivity, precision and accuracy are essential and both HPLC–MS/MRM and HPLC–MS/MRM$^3$ represent interesting options when performing analysis on a hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer. The vast majority of targeted peptide assays use HPLC–MS/MRM however interferences often occurs while performing analysis of complex biological matrices. HPLC–MS/MRM$^3$ is a technique which enables a better selectivity compared with a more conventional HPLC–MS/MRM assay and it can be performed on a hybrid QqLIT MS. This analytical approach allows the detection and quantitation by enriching standard MRM with secondary product ions that are generated within the linear ion trap. This strategy is particularly suitable when high background and/or interferences are observed in complex biological matrices (e.g. organs, plasma) and consequently standard MRM methods do not provide adequate selectivity thus affecting the assays figure of merits. Recently, a HPLC–MS/MRM$^3$ analytical method was proposed for routine analysis of low-abundance putative protein biomarkers using surrogate tryptic peptides [4]. The authors demonstrated that a technique using in silico reconstructed MRM$^3$ chromatograms using specific secondary ions produced from a trapped primary product ion, provided enhancements of the selectivity and figures of merits of the assay allowing the analysis of targeted protein biomarkers at trace concentrations in human serum. Thus, our objective was to verify if this analytical strategy could provide significant advantages over

*Abbreviations:* MRM, multiple reaction monitoring; MRM$^3$, multiple reaction monitoring cubed; HPLC, high performance liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; SP, substance P; NKA, neurokinin A; TFA, trifluoroacetic acid.

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a more traditional HPLC–MS/MRM assay for the quantification of specific tachykinin peptides in rat spinal cord.

Substance P (SP) and neurokinin A (NKA) are tachykinin peptides playing a central role in pain transmission [5]. SP and NKA are both pronociceptive peptides and agonists of the receptor neurokinin-1 (NK1) present in the lamina I of the spinal cord [6,7]. However, NKA binds preferentially to the receptor neurokinin-2 (NK2) expressed at the lower level of the spinal cord. The interaction frequency between SP, NKA and their postsynaptic receptors strongly correlates with the intensity and duration of pain [5]. Moreover, there is a close link between the up-regulation of SP and NKA with an increased release of excitatory amino acids in the intersynaptic space [8]. SP and NKA are essential elements in pain transmission and secondary hyperalgesia. SP and NKA are both important biomarkers used for preclinical pain pharmacology assessments. Consequently, the development of a selective, sensitive, precise and accurate analytical method is essential to test our hypotheses linking SP and NKA with pain behaviors.

## 2. Materials and methods

### 2.1. Chemicals

Substance P and neurokinin A were purchased from Phoenix Pharmaceutical (Belmont, CA, USA). Deuterium labeled analog peptides were synthesized and used as internal standards (CanPeptide, Inc., Pointe-Claire, QC, Canada). Acetonitrile was purchased from Fisher Scientific (NJ, USA). Trifluoroacetic acid (TFA) and formic acid (FA) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Standard solutions were prepared using a 0.25% TFA aqueous solution.

### 2.2. Sample preparation

Deuterated peptides were specifically labeled on glycine and leucine residues (SP: RPKPQQFFG($d_2$)L($d_3$)M-$NH_2$; NKA: HKTDSFVG($d_2$)L($d_3$)M-$NH_2$). They were mixed together and diluted in a 0.25% TFA aqueous solution at a final concentration of 500 ng/mL. Rat spinal cord tissues were spiked with SP and NKA at concentrations of 50, 100 and 250 pg/mg of tissues and analyzed. The calculation of the method accuracy took into account the endogenous basal concentrations and standards were prepared in an alternative solution. The most suitable solution was to use 0.25% TFA solution since this is the solution used for tissue homogenization. Standards were prepared at concentrations ranging from 20 to 500 ng/mL. To calculate the accuracy, the endogenous level needed to be subtracted from the observed concentration of fortified rat spinal cord tissues (%NOM = ([measured concentration] − [measured endogenous concentration])/[spiked concentration] × 100). Furthermore, tissue processing is an important step to preserve the sample integrity and adequately stabilize endogenous peptides. Further details can be found a previously published article [9]. The animals were euthanized with an overdose of isoflurane followed by a transection of the cervical spine. A flush of saline was performed within the spinal canal to collect the spinal cord lumbar enlargement. Tissue samples were snap-frozen in cold hexane (≈−60 °C) and stored immediately at −80 °C pending analyses. The rat tissues were weighed accurately and homogenized using a Tissue Tearor following the addition of 0.25% TFA solution at a ratio of 1:5 (w/v). Normally, the spinal cord lumbar enlargement weighs between 150 and 200 mg for animals weighing 250–350 g (7–10 weeks of age). The samples were sonicated for 20 min and 150 μL of the homogenate were mixed with 150 μL of acetonitrile to precipitate high molecular weight proteins. The samples were vortexed and centrifuged at 12,000 × *g* for 10 min. Then 150 μL of the supernatant were mixed with 150 μL of the internal standard solution (deuterium labeled peptides). The samples were then transferred into an injection vial for analysis.

**Table 1**
Selected peptide ions for the analysis in LC–MS/MRM and LC–MS/MRM$^3$ modes.

| Peptide | Precursor ions | MS$^2$ ions | MS$^3$ ions |
|---|---|---|---|
| SP | 674.7 ($z$ = 2) | 254.1 | 1086.7 |
| $d_5$-SP | | **600.3**[a] | 1171.7 |
| | | | 1199.8 |
| | 677.2 ($z$ = 2) | 254.1 | 1088.6 |
| | | **602.8**[a] | 1176.8 |
| | | | 1204.7 |
| NKA | 567.3 ($z$ = 2) | **872.4**[a] | 569.2 |
| $d_5$-NKA | | 985.6 | 716.3 |
| | | | 815.4 |
| | 569.7 ($z$ = 2) | **874.5**[a] | 569.2 |
| | | 990.5 | 716.3 |
| | | | 815.4 |

Bold values signify that this is the ion selected for MRM$^3$ analysis.
[a] Ions selected and isolated in the LIT to produce secondary product ions used to reconstruct LC–MS/MRM$^3$ ion chromatograms.

### 2.3. Bioanalytical method

The HPLC–MS/MRM and HPLC–MS/MRM$^3$ analyses were performed using a Perkin Elmer Series 200 HPLC system (Shelton, CT, USA) coupled with an AB SCIEX API 2000 QTRAP® hybrid triple quadrupole-linear ion trap mass spectrometer (Concord, Ontario, Canada). Ten μL were injected onto a Thermo Biobasic C8 100 mm × 2.1 mm (5 μm) and peptides were separated using a gradient mobile phase. The initial mobile phase condition consisted of acetonitrile (A) and water (B) both fortified with 0.4% (v/v) of formic acid and was maintained at a ratio of 5:95 (A:B) from 0 to 1 min. From 1 to 13 min a linear gradient was applied and a ratio of 60:40 (A:B) was maintained from 13 to 15 min. At 15.1 min, the mobile phase composition was reverted to 5:95 (A:B) and the column was allowed to equilibrate for 10 min for a total run time of 25 min. The flow rate was fixed at 300 μL/min and SP and NKA eluted at 13.1 and 12.4 min respectively. The eluates were analyzed using an AB SCIEX API 2000 QTRAP mass spectrometer interface with pneumatic assisted electrospray ion source. The nitrogen gas 1 was set to 25 PSI, the nitrogen gas 2 was set to 40 PSI and the electrospray electrode was set to 5000 V. The declustering potential (DP) was set to 30 V. The mass spectrometer was operated in MRM or MRM$^3$ mode using specific mass transitions with a collision energy set to 22 V. The MRM transitions were acquired with a dwell time fixed to 200 ms for each mass transition. In MRM$^3$ mode, ion trap fill time and excitation time was set to 200 and 25 ms, respectively. In all MRM$^3$ experiments, the scan rate was set to 4000 amu/s and a Q0 trapping was used. Details on selected ions used for HPLC–MS/MRM and HPLC–MS/MRM$^3$ analysis are reported in Table 1. Regression analyses were performed with PRISM (version 6.0c) GraphPad software (La Jolla, CA, USA) using nonlinear curve-fitting module with an estimation of the goodness of fit. The calibration lines were constructed from the light and heavy peptide isotopes peak-area ratios of targeted neuropeptides.

## 3. Results and discussion

Full-scan MS, MS$^2$, MS$^3$ spectra were recorded in positive mode to determine the most sensitive MRM or MRM$^3$ transitions to be used for the quantification of SP and NKA. The full-scan electrospray mass spectrum showed the formation of characteristic $[M+nH]^{n+}$ pseudo-molecular ions with the doubly charged species being the most abundant for both peptides as shown in Fig. 1A and B. MS$^2$

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