Towards Robust Quantitative MRM Plasma Analysis using Nanobore Liquid Chromatography Through Improved Nanoelectrospray Performance

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Overview

- Spray stability in nanoelectrospray can have a deleterious impact on the reproducibility of quantitative MRM-based assays.
- Here we describe the design and implementation of a Tip-Within-EmittEr Tee (TWEET) to introduce organic sheath flow to a nanobore LC column at sub-µL flow rates.
- Use of the sheath flow increased the size of the "sweet spot" of the tip position relative to the source, did not cause peak broadening and improved spray stability.

Introduction

Quantitative analysis using nanobore LC-MS/MS (nLC-MS/MS) places strict demands on nanoelectrospray MS performance metrics of sensitivity, reproducibility and robustness. Issues such as electrochemical stability of the high voltage contact and material build-up on the emitter from repeated complex sample analyses impede spray stability (Figure 1). When using a column with an integrated emitter tip, blockage or damage to the tip usually renders it unusable. Additionally, heated gases such as curtain or nebulizing gases, used to aid in electrospray stability may exacerbate the frequency of build-up.

Here we describe modifications to a nanospray source that introduce a highly optimized tip-within-emitter organic sheath flow for improved performance. Criteria evaluated include spray stability, signal intensity and, in the case of LC/MS, chromatographic peak width. The Tip-Within-EmittEr Tee (TWEET) design was implemented to gauge improvements to specific spray instabilities observed in a quantitative multiple reaction monitoring (MRM) based assay targeting 10 peptides from 7 proteins in the presence of digested plasma. One peptide, an early eluting peptide was found to be particularly sensitive to spray stability, resulting in variable peak area ratios. This peptide from Leptin, INDISHTQSVSAK, was used to gauge improvements achieved with TWEET.

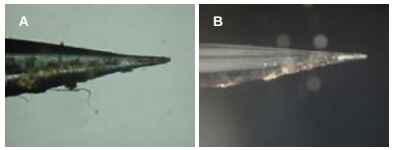

Figure 1. Photographs of nanobore columns with integrated emitter tips. Panel A: PicoFrit column with dirt and sample build-up near tip. This column analyzed 57 consecutive samples of digested plasma. Panel B: PicoFrit column (backlit) with sample deposits near tip, after 40 injections of digested plasma. Photos were taken under 10x magnification.

Methods

Mass Spectrometer

4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems) was used for all experiments. In all cases, the high voltage ESI contact was made at the distal end of the PicoFrit column with a platinum wire through a micro-tee (Upchurch).

Conventional Nanoelectrospray Tip

The customary ESI holder (Microionspray head, Applied Biosystems) holds the PicoFrit column (75 µm ID x 360 µm OD, 12 cm packed bed) and provides nebulizing gas flow around the column tip.

Methods

Tip-Within-Emitter Tee (TWEET) for Sheath Liquid

- The body of the tee is an optically clear elastomer insert with a compressible, fluoropolymer tubing core (Figure 2a) that surrounds the capillary tubing inserted into the junction. The tubing seals when external pressure from a primary nut is exerted.
- A PicoFrit column (75 µm ID x 150 µm OD, 12 cm packed bed) with integrated tip is inserted through one lateral hole and pushed through the tee into a pulled emitter tip (200 µm ID x 360 µm OD, 10 µm ID tip, Figures 2b and 2c).
- Sheath liquid is supplied through the side-arm of the tee using 50 µm ID x 360 µm OD fused silica capillary tubing.
- The blunt ends of the sheath liquid and emitter capillaries are positioned so that their edges are contiguous. Sheath liquid flows out of the side-arm and around the packed column to deliver organic solvent to the column eluate with minimal dilution.
- The sheath liquid consisted of 90% methanol/0.1% formic acid.
- Nebulizing gas was not used with the TWEET.

Direct Infusion

Nanoelectrospray stability was established by infusion of mixtures of peptides in 3% acetonitrile/0.1% formic acid and 50% acetonitrile/0.1% formic acid, peptide concentrations ~ 200 fmol/µL.

LC-MRM-MS

A 9-point standard curve (1-500 fmol/µL) was made from human plasma spiked with 7 target proteins, digested and desalted before addition of 13C/15N-labeled peptides to each sample (50 fmol/µL, digested plasma concentration = 1 µg/µL).

11 signature peptides from the 7 proteins were targeted using MRM-MS. Three transitions were monitored for each peptide and its internal standard (66 total transitions). Each sample was analyzed in quadruplicate.

An Eksigent 2D-nanoLC was used to deliver the gradient flow at 300 nL/min, and the sheath liquid flow, pumped at 5 µL/min and split to deliver ~200-300 nL/min to the tee.

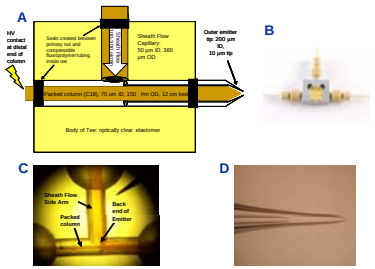

Figure 2. Representations of the Tip-within-Emitter Tee (TWEET) for Sheath Liquid introduction. **A:** Schematic showing column, emitter tip and sheath flow dimensions, not drawn to scale, within body of TWEET. **B:** Photograph of tee with capillaries and nuts. **C:** Magnified photograph of the inner junction of TWEET. **D:** Magnified photograph of the orientation of the tip-within-emitter and the low post-column volume achievable with this design. The photograph represents a 10x magnification of an empty 75 µm ID x 150 µm OD capillary with 15 µM ID tip inside a 200 µm ID x 360 µm OD, 10 µm emitter tip.

Results

TWEET Enhances Spray Stability

A mixture of trypsin digested Cytochrome C peptides was analyzed by direct infusion to determine the optimal position for the tip in front of the ion source. The tip was positioned for optimal signal in the y and z directions (up-down and towards-away from the source). The tip was then moved in millimeter increments from right to left, across the source orifice (Figure 3) until the signal was undetectable, then the tip was moved back towards the original position in 1 mm increments. The "optimal" lateral position appeared to be larger with the tee than with the conventional ESI holder. The conventional ESI holder used an empty PicoFrit column (75 µm ID x 360 µm OD, 10 µm ID tip), while TWEET employed a TaperTip (75 µm ID x 150 µm OD, 20 µm ID tip) inside the emitter (200 µm ID x 360 µm OD, 10 µm ID tip).

The source parameters for the conventional ESI holder and TWEET are shown in Table 1.

Overall spray stability was equally good between the conventional ESI holder and TWEET, when infusing samples in 50% organic solvent. The ESI voltage was set lower for TWEET, due to the higher percentage of organic solvent in the total flow introduced to the source. The curtain gas was also reduce to its lowest setting, 10. The nebulizing gas that is necessary to promote stable spray using the conventional source was not used with TWEET.

Table 1. Source Parameters for Infusion and LC/MS

Parameter	Conventional ESI holder	TWEET
ESI Voltage (kv)	2.2	1.5
Curtain Gas	20	10
Nebulizing Gas	5	Not used

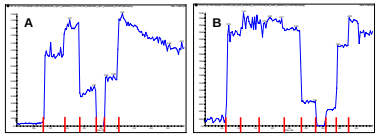

Figure 3. Total Ion Current (TIC) plots from direct infusion of 200 fmol/mL cytochrome C digest using conventional ESI tip holder and the sheath liquid tee to determine tip position for optimal ion current and stability. Panel A: Conventional ESI tip holder Panel B: Tip-within-emitter sheath flow tee. Red marks represent consecutive 1 mm movements horizontally across source opening. When signal disappeared, tip was moved back in the opposite direction.

Benefits of using TWEET for Infusion and LC/MS

- Using TWEET with the 4000 QTRAP affords the capability to infuse samples in low to no organic solvent. Samples ready for analysis by LC-MRM-MS (in initial aqueous conditions) can be directly infused without excessive optimizing of electrospray and source conditions.
- Normally, the spray stability is highly dependant upon the condition and ID of the tip used (10 mm) for optimal spray with conventional ESI holder. TWEET is less sensitive to the emitter tip condition (ie, it may be slightly chipped) and still maintains stable spray.
- Flexible use of PicoFrit or TaperFrit columns, with the tip dimensions of the column less impactful to the spray stability.

MS/MS Comparison Demonstrates Similar Intensities

Angiotensin peptide DRVYVHPF (100 fmol/µL in 50% acetonitrile/0.1% formic acid) was infused at 500 nL/min using the conventional ESI holder and the tip-within-emitter tee. Sample flow was supplied by syringe pump. Sheath liquid was supplied by Eksigent pump at ~200-300 nL/min. Precursor ion m/z 516.76 was fragmented by CID and analyzed in Product Ion mode (Figure 4). The TIC traces indicate similar spray stability between the conventional ESI holder and the sheath liquid tee. Overall signal in the product ion spectra are slightly more intense for the sheath liquid tee.

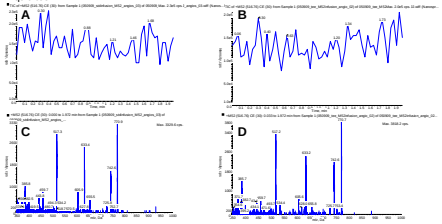

Figure 4. Total Ion Current (TIC) plots (A and B) and Product Ion scans (C and D) from direct infusion of 100 fmol/mL Angiotensin peptide using conventional ESI tip holder and the sheath liquid tee, respectively. Overall spray stability is similar with slightly increased peak intensity in the product ion scan using the sheath liquid tee (Panel D)

Better Spray Stability for Early Eluting Peptides in LC-MRM-MS

One peptide of the 11 targeted peptides in the standard curve experiment was particularly sensitive to spray stability. Leptin peptide INDISHTQSVSAK elutes at a low percent organic (10-12% acetonitrile), and often, using the conventional ESI spray holder, the spray hasn't fully stabilized. Unstable spray can affect the number of points across the chromatographic peak and ultimately, accurate quantitation when using a stable isotope labeled peptide standard. The peak area ratio for each concentration point and each of the three transitions is plotted versus concentration in Figure 5.

The range in %CV values calculated for the peak area ratio measurement (peak area light/peak area heavy) for the most intense transition ion (transition 2), based on quadruplicate sample analyses was reduced from 34-152% with the conventional ESI holder to 18-47% with TWEET.

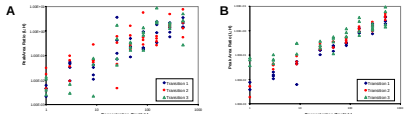

Figure 5. Log-scaled plots of peak area ratio (PAR, L:H) versus concentration for Leptin peptide INDISHTQSVSAK. The light peptide concentration range was 1-500 fmol/µL and the heavy peptide concentration was fixed at 50 fmol/µL in each sample. (A), representing the conventional ESI holder, has significantly more scatter in the PAR than the tip-within-emitter tee (B). This scatter is a result of poor spray stability, resulting in narrow peaks (<6 points across) and irreproducible peak areas.

Peak Width and Reproducibility

Peak width was not adversely affected by addition of post-column sheath liquid. Figure 6 demonstrates similar peak width in 6 extracted ion chromatograms of a single sample between the conventional ESI holder and the tee. The stability of the electrospray was also more consistent with the tee, causing fewer fluctuations in the peak shape, resulting in better reproducibility across replicate injections.

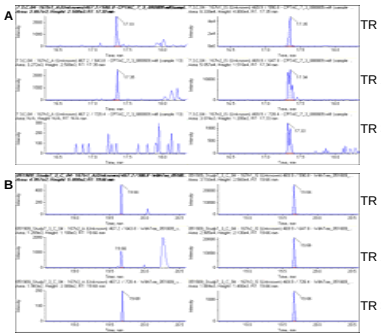

Figure 6. Extracted ion chromatograms for the 3 transitions of the light (left, 8.5 fmol/µL in 1 µg/µL plasma) and heavy (right, 50 fmol/µL), versions of Leptin peptide INDISHTQSVSAK. While peak intensity was better with the conventional ESI holder, overall peak width was similar between the conventional ESI holder (A) and the tee (B). Less noise and more consistent peak shapes were observed with the tee.

Conclusions

- A novel tip-within-emitter tee was designed for the introduction of sheath liquid, post-column, for increased spray stability.
- Use of the tee increased the "sweet spot" position of the emitter in front of the 4000 QTRAP electrospray ion source.
- While peak intensity was similar between the conventional holder and the tee, increased spray stability with the tee resulted in less variable peak areas, leading to better quantitation using isotope dilution MRM-MS on a nanoflow scale.
- Using TWEET, sample build-up at the emitter tip was greatly reduced, in part to removal of the nebulizing gas.

Funding

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