Improved Throughput for Clinical Proteomics Using a Dual Channel NanoLC-Nanospray Source

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Introduction

Liquid chromatography coupled to electrospray mass spectrometry has become an essential method for the analysis of complex mixtures of peptides. The convergence of robust nanoLC and high-resolution hybrid mass spectrometry has provided a high data dimensionality method for comparative, label-free proteomics. A major challenge in applying this MS platform to clinical proteomics is the time required to analyze individual samples. In addition to the drawbacks of a sequential method, nanoLC-MS has times in which useful data is not being acquired: during the loading of the sample, during column re-equilibration, and during times for cleaning the column to minimize carryover between samples. In order to address these issues, our laboratory is in the process of adopting a dual-column strategy. **The purpose of this study was to confirm a lack of cross-talk between channels in a dual-column nanospray source and also to determine the number of blank samples necessary to reduce carryover between samples.** The information gained from this study will be used to design and implement a dual-column workflow for LC-MS/MS analysis.

LC-MS Interface

Gradient elution LC was provided by a multi-channel nanoLC-autosampler (Eksigent) that was interfaced with an LTQ Orbitrap (Thermo). On-column injection was delivered to an automated XYZ stage nanospray source (New Objective) that was designed to hold two packed-column emitters spaced 8.9 mm apart. Separate high voltage connections based on a compressive elastomer, true zero dead volume, connectors were provided for each channel (see Figure 1). The stage position was controlled by contact closure. Continuous infusion was used for the determination of emitter cross-talk.

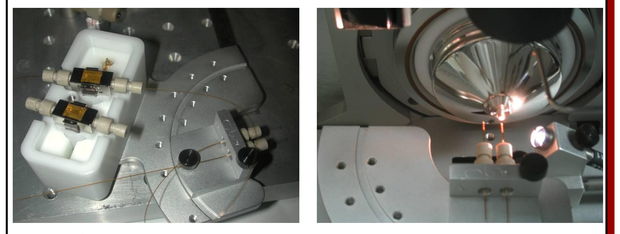

Figure 1.
Nanospray source showing high voltage connections through conductive elastomer (left) and positioning of columns relative to source on LTQ-Orbitrap (right).

Cross-Talk

A critical issue for multi-channel electrospray is signal contamination from inactive channel(s), i.e. cross-talk. For quantitative analysis the acceptable cross-talk is optimally zero. Modern mass spectrometers present a challenge in that large bore, high flow inlets have a large atmospheric volumetric capture cross section. A concentrated solution of Angiotensin (1 pmol/ul, 50% ACN, 0.1% formic acid) was introduced into the first channel (channel A) at 500 nl/min by continuous infusion. The second channel (channel B) was connected to the nanoLC. Mobile phase composition was varied from 0% to 100 ACN. With the source set to collect data from channel B, channel A sprayed continuously. The XYZ stage was used to generate a (linear) inlet-emitter map of total and analyte ion current from channel A. Within a 1 mm offset, ion intensity was unchanged, at 2 mm 90%, and 3 mm 1% and 4mm < 0.01%. With precise positioning of the channel B emitter within a 1 mm volume of the inlet using the automated XYZ stage, peptide signal from channel A was not observed (60 sec averaged acquisition) for any channel B composition. Figure 2 (below) shows the resulting plot of signal intensity as a function of distance from source.

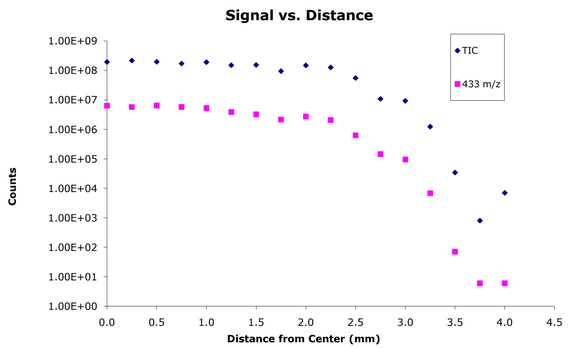

Figure 2.
Plot of signal intensity (y) vs distance from center of inlet (x). Dark blue diamonds represent total ion current; pink squares represent signal of 433 m/z.

Method: Cross talk

A concentrated solution of Angiotensin (1 pmol/ul, 50% ACN, 0.1% formic acid) was introduced into the first channel (channel A) at 500 nl/min by continuous infusion. The second channel (channel B) was connected to the nanoLC. Mobile phase composition was varied from 0% to 100% ACN. Tip position was alternated for acquisiton between Channel A and Channel B at 30 sec intervals. Figure 3 (below) shows the results of this experiment.

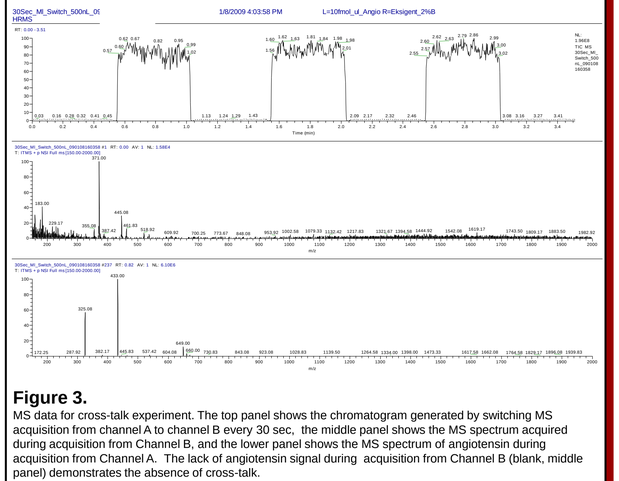

Figure 3.
MS data for cross-talk experiment. The top panel shows the chromatogram generated by switching MS acquisition from channel A to channel B every 30 sec, the middle panel shows the MS spectrum acquired during acquisition from Channel B, and the lower panel shows the MS spectrum of angiotensin during acquisition from Channel A. The lack of angiotensin signal during acquisition from Channel B (blank, middle panel) demonstrates the absence of cross-talk.

Method: Sample Carryover

In order to demonstrate degree of carryover between samples, a plasma extract was analyzed on our normal 82 min gradient. Following this, three blank samples (5 uL of water) were injected and analyzed on the same 82 min gradient. These samples were aligned in Rosetta Elucidator in order to quantify carryover.

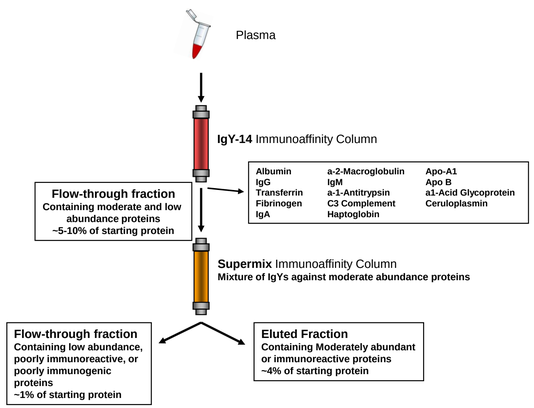

Figure 4.
Plasma depletion using two-column strategy. Depletion was carried out on 200 uL of plasma. The resulting flow-through was quantified, and 20 ug was digested in solution (LysC/trypsin) for further analysis.

Method: LC-MS/MS for Carryover Experiment

Sample analysis was performed in DDA mode with a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with a NanoLC-Ultra HPLC system (Eksigent Technologies, Dublin, CA). Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in 60% acetonitrile (B). 5.0 µL of sample was loaded over 15 min at 1.0 µL/min in 100% A onto a 20 cm x 75 µm C14 column (Jupiter Proteo, 4 µm, 90 Å, Phenomenex, Torrance, CA) and eluted at 250 µL/min using the following gradient: isocratic at 2% B (0-5 min), linear gradient from 2% B to 50% B (5-65 min), linear gradient from 50% B to 80% B (65-70 min), isocratic at 80% B (70-72 min), linear gradient from 80% B to 2% B (72-77 min), isocratic at 2% B (5 min). Total run time, including column equilibration, sample loading, and analysis was 98 min. MS analysis was performed in positive ion mode from *m/z* 350-2000 at 60,000 resolution. The following parameters were used: capillary temperature 200 ºC, source voltage 3.2 kV, source current 100 µA, tube lens 70 V, FTMS max ion time 500 msec, and FTMS MSn max ion time 1000 msec. MS/MS analysis was triggered for the six most abundant ions in each MS scan, and ions were placed on an exclusion list for 10 min after triggering 3 MS2 scans in 30 sec.

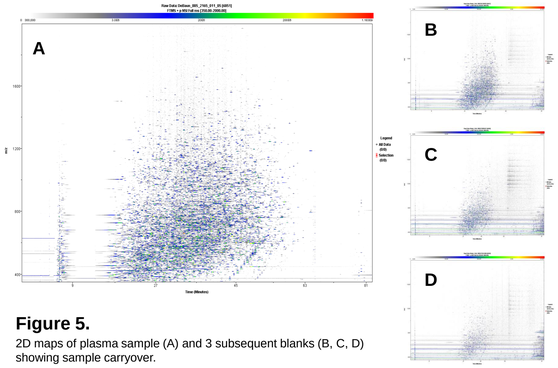

Figure 5.
2D maps of plasma sample (A) and 3 subsequent blanks (B, C, D) showing sample carryover.

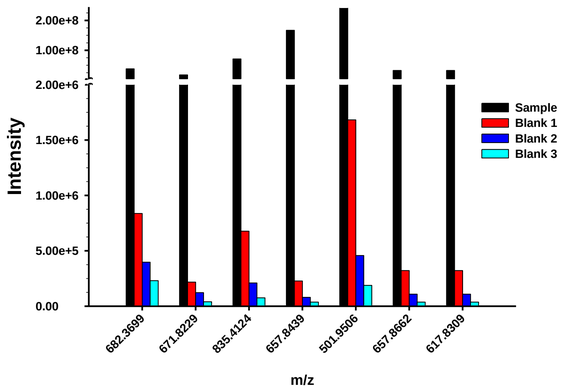

Figure 6.
Comparison of peak intensities for several plasma proteins between the original sample and three subsequent blank runs. The proteins represented in the plot above include albumin (m/z 682.3699 and 671.8229), vitronectin (m/z 835.4124, 657.8439, and 501.9506), glutathione peroxidase 3 (m/z 657.8662), and lung specific alpha-enolase (617.8309). Peak intensities were obtained from Rosetta Elucidator, and annotations were performed with the Mascot (Matrix Science) search engine through Rosetta.

m/z	Blank 1	Blank 2	Blank 3
682.3699	2.2%	1.0%	0.6%
671.8229	1.2%	0.7%	0.2%
835.4124	1.0%	0.3%	0.1%
657.8439	0.1%	0.0%	0.0%
501.9506	0.7%	0.2%	0.1%
657.8662	1.0%	0.3%	0.1%
617.8309	1.0%	0.3%	0.1%

Table 1.
Percentage of original signal intensity for each m/z value. These values were obtained by dividing the signal intensity in the relevant blank sample by the signal intensity in the original sample.

Discussion

One of our lab's primary tasks is the analysis of biological samples for the purpose of biomarker discovery. Experiments of this type involve analysis of multiple control and experimental samples by LC-MS/MS, followed by alignment of these runs in Rosetta Elucidator. Features that are identified as being significantly changed (increased or decreased) between control and experimental samples are then selected for further analysis. Therefore, signal intensity in each DDA run is critical to the success of the experiment.

Carryover studies from the plasma sample, highlighted in Figure 6 and Table 1, demonstrate the necessity for multiple blank samples between individual analytical runs. In the first blank, signal intensity is roughly 1% of that which was seen in the original run. Intensity drops to less than 1% in the second blank.

With the added blank samples, sample throughput is adversely affected. Instrument time that could be spent in spectral acquisition is instead used to clean columns for subsequent samples. Since the dual-column source has a demonstrated lack of cross-talk between Channel A and Channel B, we envision a configuration in which data acquisition occurs on Channel A while Channel B is being re-equilibrated for the next sample. Then, while data acquisition occurs on Channel B, Channel A will be re-equilibrated. In this manner, throughput is essentially doubled when compared to our current workflow. This planned workflow is outlined in Figure 7 (below).

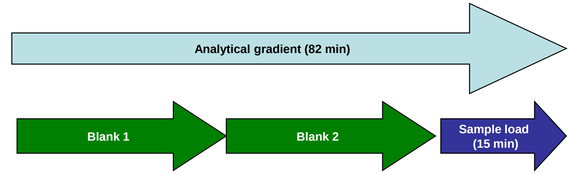

Figure 7.
Work flow using dual columns. While data analysis is being performed on one column, column cleaning and loading will be performed on a second column. At the end of the 82 min analytical gradient on the first column, the XYZ stage position will be changed, allowing for data acquisition on the second column while the first column is cleaned and loaded with the next analytical sample.

Conclusions

•The design of the automated XYZ stage nanosource eliminates cross-talk between channels, allowing for simultaneous data acquisition and column re-equilibration.

• Tryptic digest samples of plasma derived samples show significant carryover (≈ 1%) between analytical runs, leading to potential difficulties in quantitation.

• Carryover can be controlled to acceptable levels (≈ 0.1%) by running at least two blanks between samples.

Acknowledgments

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