Exploring microLC/MS/MS for Accelerating Peptide Quantitation Assays

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ABSTRACT

In this poster we will show how switching from nanoLC/MS to microLC/MS can improve throughput significantly for sensitive peptide quantitation.

INTRODUCTION

A common view of the protein/biomarker research pipeline is that one starts with high sensitivity global discovery at nanoflow rates and then proceeds to higher throughput targeted quantitation on a smaller number of targets. To confirm or refute their ultimate utility, many more samples must be analyzed with increased throughput and robustness, which means faster chromatography and/or higher flow rates. At higher flow rates sensitivity may decrease due to reduced ionization efficiency compared to nanospray ionization. There has been increased interest in working in the microflow regime (3-50 µL/min) to obtain a good balance between throughput, robustness and sensitivity. In this study, we will introduce microLC methods for high throughput , high sensitivity peptide quantitation.

MATERIALS AND METHODS

HPLC

An Eksigent microLC 200 or nanoLC 425 system in micro-mode was used. All trap-elute experiments were performed using the second gradient or loading pump that is part of the nanoLC 425 system. Mobile phase A was water with 0.1% formic acid and B was acetonitrile with 0.1% formic acid. An Eksigent cHiPLC system was used for the nanoLC experiments. See text and figures for columns, gradients, flow rates and injection volumes used.

MS

An AB Sciex QTRAP® 5500™or TripleTOF® 5600 system with Turbo V™ source or NanoSpray® III ion source was used. The electrode in the Turbo V™ source was changed to a microLC hybrid electrode with 25 µm ID (Eksigent). Data was processed with MultiQuant™ Software to perform quantification and statistical analysis.

Sample

Samples used were a beta-Galactosidase (BG) tryptic digest (AB SCIEX), a synthetic peptide standard with isotopically labeled peptides (CYP450 assay kit, AB SCIEX), a six protein mix digest (Michrom Bioresources), Bradykinin (Sigma) and exanatide/isotope labeled exanitide (GSK)

RESULTS

Comparison nanoLC-microLC

A comparison of the relative sensitivity between nanoLC/MS and microLC/MS was made using a selection of ten tryptic peptides from the Michrom six protein mix digest. We compared both 75 µm and 200 µm ID chip nanolc columns with 0.3 and 0.5 mm ID columns. Sensitivity only decreased by a factor 4 going from a 75 µm ID chip column run at 300 nL/min to a 0.5 mm ID microLC column at 10 µL/min (see table 1). Throughput could be increased by almost a factor 2, because of less delay time and less post-column dispersion.

Column ID	Flow Rate	Source	LLOQ* increase relative to 75 µm
75 µm	300 nL/min	NanoSpray® Source	1
200 µm	1.0 µL/min	NanoSpray® Source	2.5
300 µm	4 µL/min	Turbo V™ Source	3
500 µm	10 µL/min	Turbo V™ Source	4

* LLOQ defined as lowest point on concentration curve with <20 %CV precision and accuracy between 80 and 120%; S/N >10

Table 1. Comparison of Sensitivity Differences using different column ID's and flow rates. The LLOQ difference for each peptide at each column diameter was computed relative to the LLOQ for the 75 µm ID column, then this difference was averaged across the 10 peptides monitored. These results should provide a rough guidance to use when trying to decide which column and flow rate will be best suited for the experiment planned.

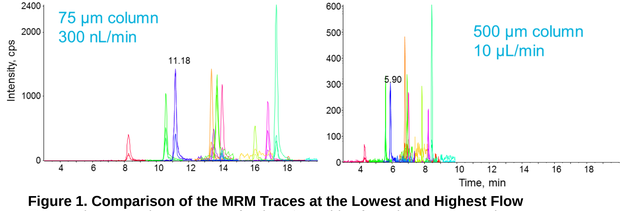

Figure 1. Comparison of the MRM Traces at the Lowest and Highest Flow Rates. The MRM chromatogram for the 10 peptides from the 75 µm ID column was obtained using a 15 min linear gradient, peptides are observed from 8 – 18 mins (top). At the higher flow rates, faster gradients can be used. Analyzing the same peptides on a 500 µm ID column shows a small decrease in sensitivity but an increase in throughput (7 minute linear gradient). However equivalent resolving power was obtained.

High Throughput with direct injection

Further throughput improvements were explored using a 0.3 x 50 mm column packed with a superficially porous phase optimized for peptide analysis (HALO peptide). This type of phase is designed to be operated at very high linear flow rates while maintaining efficiency. We used a flow rate of 30 µL/min and a gradient from 10-40%B in 2 min. A sample with 28 labeled CYP450 labeled tryptic peptides (AB SCIEX) was analyzed with 3 MRM transitions per peptide. As the peak widths were in the order of seconds, the transitions were scheduled in order to ensure a sufficient number of data points across each peak were acquired. This type of ultra-fast separations is especially useful when there are only a limited number of peptides in the sample, e.g. samples from a SISCAPA (**S**table **I**sotope **S**tandards and **C**apture by **A**nti-**P**eptide **A**ntibodies) workflow.

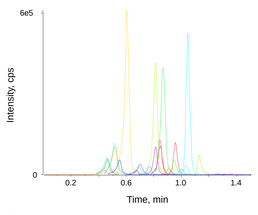

Figure 2. Separation of 28 tryptic peptides in less than 1.5 min.

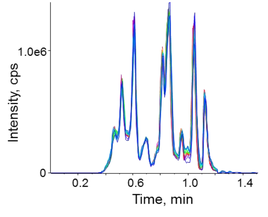

Figure 3. TIC Overlay of 20 replicate injections. Average CV per MRM is 7.7%

Trap-elute

On-line solid phase extraction ("trap-elute") workflows allow for injecting larger volumes of sample while maintaining throughput, as the sample can be loaded on the trap column at a much higher flow rate that the flow rate used for the separation. Additional advantage is that any salts and other components not retained on the trap columns are washed away to waste, and can't contaminate the separation column and/or MS. The set-up is illustrated in figure 4 below. Fig 5. illustrates the sensitivity gain that can be achieved loading larger sample volumes of Bradykin in crashed plasma using an Eksigent 0.3 mm x 15 cm ChromXP™ C18 3 µm column (Eksigent) operated at 4 uL/min ,and a 0.3 x 10 mm C18 trap (Dr. Maisch, Germany) loaded at 20 µL/min.

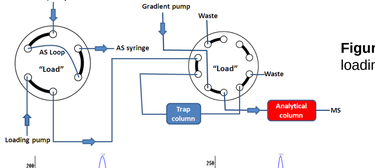

Figure 4. Trap-Elute set-up for fast loading of larger sample volumes

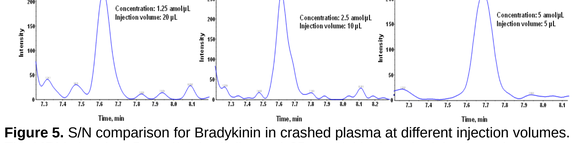

Figure 5. S/N comparison for Bradykinin in crashed plasma at different injection volumes. The MRM signal at 5 amol/µL is similar as 1.25 amol/(µL) when the injection volume was increased from 5 µL to 20 µL

High Throughput with Trap-Elute

As a final example we developed a fast trap-elute method for the quantitation of Exanatide, a 39-amino acid peptide drug for the treatment of type 2 diabetes. A 0.5 x 50 mm column packed with 3 µm C18 phase (ChromXP C18CL, Eksigent) was used with a 0.5 x 5 mm trap packed with 5 µm C18 (Thermo Scientific).
Flow rate for the separation was 40 µL/min with a gradient from 5-90%B in 1.5 min, while sample loading was performed at 50 µL/min for 1.5 min. 50 µL of sample was loaded and the trap was washed off-line from the column with 50/50 acetonitrile/isopropanol with 1% Trifluoroethanol and 0.1 % Formic acid to reduce carry-over (see figure 6). The method was fast, cycle time less than 10 min., sensitive (LLOQ of 2 pg/mL) and accuracy, precision and linearity were comparable with conventional LC methods (see Table 2.).

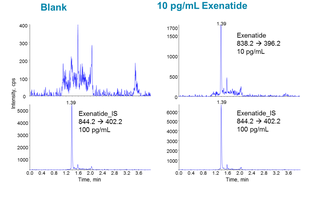

Figure 6. MRM chromatogram for matrix blank with IS and 10 pg/mL Exenatide showing minimal carry-over.

Actual Concentration (pg/mL)	Mean (pg/mL)	Standard Deviation (n=4)	Percent CV
2	2.070	0.1427	6.89
5	5.292	0.3281	6.20
20	20.08	1.278	6.37
50	50.52	2.274	4.50
200	183.2	3.764	2.05
500	468.1	14.34	3.06
1000	1028	42.79	4.16
2000	2029	49.62	2.45
5000	4988	207.2	4.15

Table 2 . Showing <7 % CV and Accuracy between 90 and 110% with an LLOQ of 2 pg/mL. The method is linear of > 3 orders of magnitude.

CONCLUSIONS

We have shown that moving from nanoLC/MS to microLC/MS for peptide quantitation can significantly increase throughput while sensitivity is only minimally reduced. Trap and Elute type workflows allow for injecting larger sample volumes without increasing the cycle time of the assay.

REFERENCES

1AB SCIEX Technical note "Exploring the Sensitivity Differences for Peptide Quantificiation in the Low Flow Rate Regime
2AB SCIEX Technical note "On-line Pre-concentration with MicroLC for Peptide Quantitation".

TRADEMARKS/LICENSING