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Quantitative improvements in peptide recovery at elevated chromatographic temperatures from μ LC/MS analyses of brain using SRM mass spectrometry

Santiago E. Farias^{1,*}, Kelli G. Kline^{2,*}, Jacek Klepacki^{1,*}, and Christine C. Wu^{1,#}

¹ Department of Pharmacology, University of Colorado School of Medicine, Anschutz Medical Campus, Aurora, Colorado, 80045

² Genetics and Biotechnology Center, University of Wisconsin, Madison, WI, 53706

Abstract

Elevated chromatographic temperatures are well recognized to provide beneficial analytical effects. Previously, we demonstrated that elevated chromatographic temperature enhances the identification of hydrophobic peptides prepared from enriched membrane samples. Here, we quantitatively assess and compare the recovery of peptide analytes from both simple and complex tryptic peptide matrices using the SRM mass spectrometry. Our study demonstrates that elevated chromatographic temperature results in significant improvements in the magnitude of peptide recovery for both hydrophilic and hydrophobic peptides from both simple and complex peptide matrices. Importantly, the analytical benefits for quantitative measurements in whole mouse brain matrix are demonstrated, suggesting broad utility in the proteomic analyses of complex mammalian tissues. Any improvement in peptide recovery from chromatographic separations translates directly to the apparent sensitivity of downstream mass analysis in μ LC-MS/MS based proteomic applications. Therefore, the incorporation of elevated chromatographic temperatures should result in significant improvements in peptide quantification as well as detection and identification.

INTRODUCTION

One of the major limitations for quantitative proteomic analyses of complex biological samples is low analytical throughput. Complex samples (such as mammalian tissues) are typically fractionated to enrich for low abundance proteins^{1–3}. However, fractionation increases the total number of samples to be analyzed, requires larger amounts of input tissue, and may lead to errors associated with reproducibility of fractionation and biased biochemical losses. Optimal throughput is achieved by bypassing the fractionation step. However, quantitative measurements in complex unfractionated tissue samples necessarily require improvements in analyte separation, recovery and detection. Many current proteomic platforms are μ LC-MS/MS based, and improvements in instrument performance, sensitivity, and analytical speed, are continuously being realized⁴. Likewise, improvements in μ LC separations and chromatographic recovery can also contribute significantly to the throughput of analyses by enhancing the “apparent” sensitivity of the analysis.

[#]Corresponding Author: Christine C. Wu, Ph.D., Assistant Professor of Pharmacology, University of Colorado School of Medicine, Department of Pharmacology, Mail Stop 8303, RC1-South Tower, L18-6117, 12801 East 17th Avenue, PO Box 6511, Aurora, CO 80045, Office: 303-724-3351, FAX: 303-724-3663, christine.wu@ucdenver.edu.

*These authors contributed equally to this work.

It is well recognized that conducting liquid chromatography (LC) at elevated temperatures (typically 30–80°C) results in beneficial effects, such as improvements in peak shape, selectivity, and resolution^{5,6}. Furthermore, elevated temperatures are sometimes required for the successful elution of very hydrophobic proteins⁷. Previously, we demonstrated that elevated chromatographic temperature enhances the recovery, and therefore the identification, of hydrophobic peptides from enriched plasma membrane samples using undirected μ LC-MS/MS analyses⁸. This study utilized peptide identification from tandem mass spectra acquired from shotgun analyses as the metric for improvements in total peptide recovery. However, quantitative measurements for each peptide analyte were not assessed and the magnitude of peptide recovery was not determined. In this study, we used targeted mass spectrometry to quantify the detection of 18 synthetic peptides of a wide range of hydrophobicities when added to both a simple peptide matrix (derived from 5 proteins) and a complex peptide matrix (derived from total mouse brain) during standard reversed-phase chromatography conducted at 25°C, 40°C, and 60°C. Our results demonstrate that increased chromatographic temperature results in significant improvements in peptide recovery for all 18 peptides (hydrophilic and hydrophobic) monitored in both the simple and complex peptide matrix. On average, peptide recovery increased by 5-fold and 10-fold in the low and high complexity matrices respectively at elevated chromatographic temperatures. Specifically, the improvements demonstrated in the whole brain peptide matrix have important implications regarding the utility of this approach for complex biological applications. Because improvements in peptide recovery from chromatographic separations translate directly to improvements in mass spectrometric analyses for μ LC-MS/MS based proteomic applications, the simple incorporation of elevated chromatographic temperatures will provide significant improvements in peptide quantification, as well as detection and identification, in complex mammalian tissues.

METHODS

Reagents

All solvents were purchased from Fisher Scientific (Pittsburgh, PA), and all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Synthetic peptides with crude purity (< 70%) were purchased from Thermo Scientific (Huntsville, AL).

Preparation of peptide matrices

Two tryptic peptide matrices of low and high complexity were prepared as follows:

Low complexity matrix

A tryptic digest of an equimolar mixture of the five yeast proteins (alcohol dehydrogenase, enolase, triosephosphate isomerase, hexokinase, and phosphoglucose isomerase) was purchased from Michrom Bioresources, Inc. The lyophilized tryptic peptides were diluted in 95% H₂O, 5% acetonitrile, 0.1% formic acid to a final concentration of 100 fmol/ μ l.

High complexity matrix

Whole brains (n=2) were collected from male C57BL/6J mice after cervical dislocation. Brains were dissected into left and right hemispheres, flash frozen in liquid nitrogen, and stored at –80 °C until use. Half brains were homogenized in 800 μ l ice-cold homogenization buffer (1X PBS, 400 nM aprotinin, 1 mM AEBSF) using a tissue tearor homogenizer with a 4.5 mm inner diameter probe (Biospec Products, Bartlesville, OK). The sample was centrifuged at 420 g for 10 min at 4°C to pellet nuclei, unbroken cells, and cell debris. The supernatant was collected and the protein concentration was determined by BCA protein assay. The supernatant (100 μ g protein) was subjected to a chloroform/methanol extraction as previously described⁹, and the resulting protein precipitate was solubilized in 0.1% RapiGest (Waters Corp, Milford, MA)

in 25 mM ammonium bicarbonate. Solubilization of the protein pellet was assisted using a Microson Ultrasonic cell disruptor (Misonix, Framingham, MA; 10 s, power 10) and passing the solution through an insulin syringe (1 cc, 28 gauge) approximately ten times. The solubilized protein sample was then boiled for 2 min. Dithiothreitol (DTT, 5 mM final concentration) was added, and samples were incubated at 60°C for 30 min to reduce protein disulfide bonds. After the sample was cooled to room temperature, iodoacetamide (IAA, 15 mM final concentration) was added and samples were incubated at room temperature for 30 min in the dark to alkylate reduced cysteines. Modified trypsin (Promega, Madison, WI) was added at a 1:100 enzyme/substrate with CaCl₂ (1 mM final concentration) and incubated at 37°C overnight using a thermomixer (Eppendorf, Westbury, NY). The digestion reaction was terminated by storing the samples at -20°C. To hydrolyze the RapiGest prior to mass spectrometry analysis, HCl was added to a final concentration of 100 mM, and the solution was incubated at 37°C for 45 min using a Thermomixer, and then centrifuged at 20,800 g at 4°C for 15 min.

Addition of synthetic peptides to peptide matrices

Eighteen synthetic peptides with GRAVY (grand average of hydropathy) scores (calculated as previously described¹⁰) ranging from -0.82 to +1.57 and corresponding to endogenous proteins from mouse brain were selected for analysis (Table 1). The peptides were combined in an equimolar mixture and added to both the low and high complexity matrices (described above) to achieve a final concentration of 50 fmol/μl for each peptide.

Thermostatted microcapillary liquid chromatography (μLC) coupled with selective reaction monitoring (SRM) mass spectrometry

A ThermoElectron MicroAS autosampler/Surveyor HPLC pump system and TSQ Quantum Access mass spectrometer (Waltham, MA), running Xcaliber software, were used for all sample analyses. With the use of the autosampler as previously described¹¹, samples (5 μl low or high complexity peptide matrix spiked with synthetic peptide mixture) were loaded onto a fused silica (Polymicro Tech, Phoenix, AZ) microcapillary column (75 μm i.d. with a 5 μm pulled tip) packed with 15 cm of 5 μm 125 Å Aqua C18 reversed-phase (RP) material (Phenomenex, Torrance, CA). The microcapillary column temperature was controlled by a column heater built in-house as previously described¹². The μLC was carried out using an increasing gradient of aqueous acetonitrile (MeCN) containing 0.1% formic acid over a 65 min run, consisting of a 15 min loading step at 4.2% MeCN, followed by a gradient up to 30% over 30 min, followed by a gradient up to 70% over 10 min, followed finally with an equilibration step again at 4.8% for 10 min.

The doubly charged precursor ion and the two most intense singly charged product y-ions for each synthetic peptide (Table 1) were monitored in SRM mode. The data was acquired with a Q1 and Q3 resolution of 0.7 *m/z*. Each transition was monitored with a dwell time of 50 ms resulting in a total cycle time of 1 s. The RF-only q2 collision cell was pressurized with 1 mTorr of argon gas and collision energies of the transitions monitored ranged from 24 to 36V. The samples were analyzed with the reversed-phase column maintained at 25°C, 40°C and 60°C, with five technical replicates performed per given temperature.

The temperature sequence of the replicate μLC-MS/MS analyses was randomized (Table S-1). Peptide abundance is reported as the sum of the area under the curve (AUC) for the two precursor-to-product ion transitions monitored for each peptide using Skyline v0.5 software¹³ while maintaining a constant column back pressure. The FWHM of fifteen peptides was calculated using Skyline v0.5 software¹³ in experiments where the flow rate through the column was maintained at 0.5 μl/min at all experimental temperatures by adjusting the length of the waste split to account for decreasing solvent viscosity at increasing temperature.

Statistical analysis

Peptide recoveries (Figures 2 and 3) are reported as the AUC mean $\pm$ standard error of the mean (SEM). An unpaired two-tailed *t*-test was used to compare means between $^{*}25^{\circ}\text{C}$ – 60°C , $^{+}25^{\circ}\text{C}$ – 40°C and $^{\circ}40^{\circ}\text{C}$ – 60°C using GraphPad Prism version 4.0b (GraphPad Software, San Diego, CA, www.graphpad.com). Statistical significance was taken as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ to compare means between 25°C – 60°C ; $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{+++}p \leq 0.001$ to compare means between 25°C – 40°C ; and $^{\circ}p \leq 0.05$, $^{\circ\circ}p \leq 0.01$, $^{\circ\circ\circ}p \leq 0.001$ to compare means between 40°C – 60°C . Peptide peak widths (Figure 4) are reported as the mean FWHM $\pm$ SEM for each peptide. A paired two-tailed *t*-test was used to compare the means between $^{*}25^{\circ}\text{C}$ – 60°C , $^{+}25^{\circ}\text{C}$ – 40°C and $^{\circ}40^{\circ}\text{C}$ – 60°C using GraphPad Prism, Version 4.0b (GraphPad Software, San Diego, CA, www.graphpad.com), with statistical significance taken as previously described (reference). Peak capacity is reported as the gradient run time divided by the average peak width (FWHM) at each temperature ^{14,15}.

RESULTS AND DISCUSSION

Previously, we reported that elevated temperature during standard reversed-phase chromatography is required to maximize the identification of hydrophobic peptides and integral membrane proteins ¹². The study characterized the benefits of temperature by comparing the observed identified peptides in total number and hydrophobicity from two selected samples consisting of enriched integral membrane proteins. However, quantitative metrics associated with each peptide analyte were not determined and improvements in peak capacity were not assessed. These measurements are crucial to assess the benefits of elevated chromatographic temperature for quantitative proteomic approaches, particularly those involving SRM analyses. Therefore, in this study, targeted SRM measurements were acquired for 18 peptides added to two sample matrices to compare the effect of chromatographic temperature on the quantitative recovery of each peptide analyte (Table 1). The selected peptides had an average length of 11 amino acids, were derived from native mouse brain proteins, and represented a wide range of GRAVY scores, from -0.82 to $+1.57$. In general, positive (+) GRAVY scores are associated with peptides with overall hydrophobic character, whereas negative (–) GRAVY scores indicate hydrophilic character. An equimolar mixture of these peptides was added to each of the two sample matrices, selected to determine if there are differences associated with matrix complexity. The low complexity matrix was a tryptic digest of five yeast proteins, and the high complexity matrix was a tryptic digest of whole mouse brain.

Comparison of peptide retention time with temperature

The 18 synthetic peptide analytes were monitored by SRM analysis in the context of both the low and high complexity matrices during separation on a standard RP microcapillary column, maintained at 25°C , 40°C , and 60°C and placed in-line with a ThermoElectron Quantum Access mass spectrometer. Previous reports have shown that peptide retention times are affected by temperature ^{6,8,16,17}. Increased temperature decreases peptide elution time due to increased mass transfer between the stationary and mobile phases, and because of decreased mobile phase viscosity and increased solubility of the solute in the mobile phase ^{6,16}. Therefore, it was important to characterize the retention time shifts for all selected peptides to ensure that they were kept within the optimal chromatographic separations range for the comparison of quantitative measurements.

As shown in Figure 1, peptide retention time decreased as column temperature increased. The temperature dependent peptide retention time shift was similar for all selected peptides in both the low (Figure 1a) and the high (Figure 1b) complexity matrices. In the low complexity matrix the average peptide retention time shift from 25°C to 60°C was 3.97 min, while the same peptides in the high complexity matrix displayed a slightly higher average shift of 4.33 min.

An example of this retention time shift is shown for a unique peptide from the Gria-2 protein in both low (Figure 1c) and high (Figure 1d) complexity matrices. This trend for the increased magnitude of retention time shift for a given peptide analyte in the higher complexity matrix is an empirical observation for all samples analyzed to date (data not shown).

Effect of temperature on peptide recovery in low complexity matrix

Peptide recovery was determined by measuring the area under the curve of the two most intense precursor-to-product ion transitions monitored for each peptide. In the low complexity matrix, 13 out of the 18 synthetic peptides tested (72%) showed a significant improvement in recovery at higher temperatures (Figure 2). Ten peptides had maximal recovery at 40°C with a recovery plateau at 60°C. Only three peptides, DAT-4 (GRAVY score 1.57), Gnm-M3 (GRAVY score 1.40), and Homer-21 (GRAVY score 0.69) showed optimal recovery at 60°C. On average, a five-fold increase in peptide recovery was observed when the temperature was elevated from 25°C to 40°C, and a four-fold increase from 25°C to 60°C.

Effect of temperature on peptide recovery in high complexity matrix

In the high complexity matrix, all 18 peptides (100%) had a significantly improved recovery at higher temperatures (Figure 3). Thirteen peptides (72%) had maximal recovery at 40 °C with a recovery plateau at 60°C, while five peptides (28%) had optimal recovery at 60 °C. On average, a ten-fold increase in peptide recovery was observed for the 18 peptides when moving from 25°C to 40°C, and a nine-fold increase from 25°C to 60°C. The overall peptide SRM response was higher in the low complexity matrix when compared to the high complexity matrix as determined by the AUC, possibly due to higher ion suppression effects in the high complexity matrix. In addition, the average fold of increase in peptide recovery was greater in the high complexity matrix (10 and 9-fold increase for 25°C to 40°C and 25°C to 60°C temperature increases, respectively) than the low complexity matrix (5 and 4-fold increase for 25°C to 40°C and 25°C to 60°C temperature increases, respectively). The alteration of peptide elution time in the complex matrix may change the environment in which each peptide is eluting, and therefore can alter the matrix effects the peptide will experience. This may allow for a greater increase in recovery in the high complexity matrix compared to the simple mix where ion suppression/matrix effects likely do not have as large an impact.

Effect of temperature on chromatographic resolution

The beneficial effects of elevated temperature on peak resolution are well established, and previous reports have shown elevated temperatures are useful in decreasing overall analysis time while enhancing peak shape and selectivity^{18,19}. In this study it was found that increasing μ LC column temperature positively influenced peak resolution as determined by peptide FWHM. Increasing μ LC temperature significantly decreased overall peptide FWHM in both low (Figure 4a) and high (Figure 4b) complexity matrices. In both high and low complexity matrices FWHM significantly decreased when column temperature was changed from 25°C to 60°C and from 40°C to 60°C. In these studies, the mobile phase flow at the μ LC column tip was maintained at a constant rate at 0.5 μ l/min, resulting in decreased backpressure at increased temperatures; therefore, the decreased peptide FWHM should not be due to the influence of increased flow at elevated temperatures. We did not observe a significant difference in peak width between the hydrophilic and hydrophobic peptide species in either matrix. Peak capacity is defined as the number of peaks that can be separated in a given retention window, and is generally used as the best measure of the performance of a gradient separation (reviewed in⁽¹⁴⁾). In the low complexity matrix, the peak capacity increased from 419 (at 25°C) to 461 (at 40°C) to 508 (at 60°C), resulting in a 10% overall increase in capacity. Similarly, in the high complexity matrix, peak capacity increased from 383 (at 25°C) to 432 (at 40°C) to 467 (at 60°C), yielding an overall 16% increase. These results indicate that elevated chromatographic

temperature decreases peak width and increases column efficiency for the analysis of these peptide analytes, particularly in the high complexity matrix (See Table S-2).

CONCLUSION

Elevated chromatographic temperatures during μ LC-MS/MS based analyses using standard RP columns quantitatively improves peptide recovery in both low and high complexity matrices. Previously our group highlighted the benefits of elevated temperature for the detection of hydrophobic peptides in undirected MS analyses. This report expands upon that finding and demonstrates by targeted SRM analysis that improved recovery can be achieved for peptides across a range of hydrophobicities by using elevated chromatographic temperatures. Importantly, this effect was observed to lead to dramatic improvements in the detection of peptide analytes prepared from unfractionated whole brain samples. Temperature control devices for μ LC systems are commercially available, easy to incorporate, and should be considered for routine use. Because improvements in peptide recovery from chromatographic separations translate directly to the apparent sensitivity of downstream mass analysis in μ LC-MS/MS based proteomic applications, the incorporation of elevated chromatographic temperatures should result in significant improvements in peptide quantification as well as detection and identification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of retention time for at different chromatographic temperatures in low (a) and high (b) complexity matrices. Gria-2 peptide (transitions m/z 719→793 and 719→892) retention time shift at different temperatures in low (c) and high (d) complexity matrices.

Figure 2.

Recoveries observed for 18 synthetic peptides added to a low complexity matrix (tryptic digest of 5 yeast proteins) at 3 chromatographic temperatures. Bars are displayed in order of increasing temperature clustered for each peptide identified by GRAVY score.

Figure 3.

Recoveries observed for 18 synthetic peptides added to a high complexity matrix (tryptic digest of whole mouse brain) at 3 chromatographic temperatures. Bars are displayed in order of increasing temperature clustered for each peptide identified by GRAVY score.

Figure 4.

Elevated chromatographic temperature decreased average peptide peak width in both low (a) and high (b) complexity matrices. Peptide peak widths are reported as FWHM measurements (calculated using Skyline) and the average measurement for all peptides monitored is plotted for each temperature.

Table 1

Selected synthetic peptides (summary of parent mouse protein, GRAVY score, peptide sequence, precursor m/z , SRM transitions monitored).

Mouse Protein	GRAVY	Peptide Sequence	m/z	Transition 1	Transition 2
Ce63-9	-0.82	LLEALQEEQK	600.83	600.83/974.48	600.83/845.44
Ppm1-b1	-0.69	YGLSSMQGWR	592.78	592.78/851.38	592.78/764.40
Homer-20	-0.57	MGEQPIFTTR	590.30	590.30/734.42	590.30/524.28
Paqr7-1	-0.46	PEPVFTVDR	530.28	530.28/833.45	530.28/637.33
P2RX4-2	-0.39	AFLFEYDTPR	629.81	629.81/927.42	629.81/780.35
Gria-1	0.07	AWNGMVGEIYYGR	726.36	726.36/793.42	726.36/892.49
Homer-26	0.27	SFLEVLGDK	504.27	504.27/773.44	504.27/660.36
Homer-6	0.42	VIINSTITPNMTFTK	840.46	840.46/939.46	840.46/838.41
Homer-2P	0.50	IALTQSAANVK	558.32	558.32/818.43	558.32/589.33
FABP3-2	0.64	SLGVGFATR	454.25	454.25/707.38	454.25/551.29
Homer-21	0.69	QAVTVSYFYDVTR	774.89	774.89/1050.49	774.89/1250.61
Rdh11-2	1.07	LANILFTK	460.28	460.28/735.44	460.28/806.48
DAT-2	1.32	IDFLLSVIGFAVDLANVWR	1074.60	1074.60/1247.65	1074.60/1360.74
Gria2	1.36	ADVAVAPLTITLVR	719.94	719.94/912.59	719.94/983.62
Gnmt-M3	1.40	AWLLGLLR	471.30	471.30/684.48	471.30/571.39
Rdh11-4	1.41	WLWQLFFVFIK	763.93	763.93/913.55	763.93/1227.69
FABP3-3	1.48	LILTLTHGVSIVSTR	748.00	748.00/705.38	748.00/842.44
DAT-4	1.57	PYVVLTAALLR	629.40	629.40/898.61	629.40/799.54