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Advanced Biotransform Solution: Sensitive Microflow Analysis of Peptide Catabolism

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Abstract

Therapeutic peptides are highly potent, selective, relatively safe and well tolerated by humans which differentiates them from traditional small molecule drugs. Based on recent data, more than 60 peptide drugs have reached the market for treatment of a wide range of diseases including bone and prostate cancer, type 2 diabetes, osteoporosis, heart failure and several others-with increasing market potential evidenced by ~140 peptide therapeutics in clinical trials and more than 500 therapeutic peptides in preclinical development[1]. Therapeutic peptides are susceptible to proteolytic cleavages by serum and tissue proteases and potential biotransformation of their amino acids. Hence, there is a growing need for identifying every detectable metabolite or peptide catabolite during drug development and to monitor peptide biotransformations across the experimental sampling interval. Advancements in Liquid Chromatography (LC) coupled to Tandem Mass Spectrometry (MS/MS) have enabled comprehensive identification of peptide catabolites in a complex system such as serum or plasma, which is critical during drug discovery and development. The purpose of this study was to develop an integrated microflow LC-MS/MS solution coupled to the SCIEX Advanced Biotransform Solution[2] to take advantage of the gains in sensitivity from microflow LC and to identify peptide catabolites at low levels. The increased sensitivity is critical when the sample volume is limited as in small animal studies.

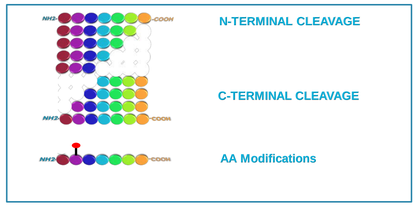

Figure 1. Peptide Biotransformation. Amino acids modifications, N-Terminal or C-Terminal cleavages of peptides by active proteases and enzymes in blood or tissue upon therapeutic peptide administration are major peptide catabolism products often need to be analyzed by LC-MS/MS.

Materials and Methods

Sample Preparation: Three therapeutic peptides (human parathyroid hormone fragment 1–34 (PTH) , human calcitonin gene related peptide (CGRP) and porcine tetradecapeptide renin substrate 1-14 (Renin)), obtained from Sigma-Aldrich were spiked in 50 µl of rat plasma (50 µg/mL) and as a control in HPLC grade water, then incubated at 37˚ C for 10, 30 and 60 minutes. After incubation, proteins were precipitated with the addition of 200 µL of cold acetonitrile (30 min at 4˚ C), then centrifuged at 14000 RPM for 15 minutes. 200 µL of supernatant was transferred to a clean tube and dried using a TurboVap evaporator using no heat. Pellet was reconstituted in 1000 µL of 2% acetonitrile in 0.1% formic acid in water prior to the LC-M/MS analysis. 50 µL of rat plasma spiked with water was prepared under same condition, as control plasma sample.

Analytical Flow Liquid Chromatography: A Shimadzu Prominence HPLC system with two LC-20AD pumps, CTO-20A column oven and a SIL-20AC autosampler was used for analytical flow LC-MS/MS analysis. The column used was a 100 x 2.1 mm Kinetex C18 2.6 µm 100 Å column (Phenomenex). Mobile phase A, water with 0.1% formic acid, and mobile phase B, acetonitrile with 0.1% formic acid was used at a flow rate of 350 µl/min. Wash solvent for the autosampler was 20/20/60 methanol/acetonitrile/IPA. Injection volume was 5 µL, and the column was kept at 35˚ C. The LC gradient method used for both analytical and microflow LC is listed in Table 1.

Time	%B
0	3
1	5
1.5	10
4.0	32
9	40
9.5	95
11	95
11.1	3
15	3

Table1. The LC gradient used for traditional and microflow LC-MS.

Microflow Liquid Chromatography: A SCIEX M3 MicroLC system, with an integrated autosampler, was used in direct injection mode in combination with a source mounted column oven (SCIEX). A 50 x 0.3 mm HALO Peptide ES-C18 2.7 µm 160 Å column was used (SCIEX). Mobile phase A, water with 0.1% formic acid, and mobile phase B, acetonitrile with 0.1% formic acid was used at a flow rate of 10 µl/min. The column temperature was set to 35˚ C. Injection volume was 5 µL, and the autosampler needle and valve wash consisted of 1 cycle using mobile phase B, followed by two cycles using mobile phase A.

MS/MS Conditions: For the microflow LC experiments, the standard electrode (100 µm ID) was replaced with a 25 µm ID electrode with DuoSpray™ Source and TripleTOF® 6600 (SCIEX). LC-MS/MS data was collected with IDA (m/z 300-1600 ,Top 10, CE 40+/- 5) and SWATH® acquisition (m/z 300-1600, SWATH with fixed window of 50 Da, optimized source parameter were used for each LC flowrates[3].

Data Analysis: MetabolitePilot™ 2.0 [3,4,5] was used for peptide catabolite identification and PeakView® 2.2 software was used to compare the sensitivity of the microflow and analytical flow LC-MS/MS data for the peptide's top three catabolites.

Results

Most LC-MS/MS methods for biotransformation studies use analytical flowrates of 350-500 µl/ min to profile peptide catabolites and often lack required sensitivity to identify low level catabolites. The microflow LC method at 10 µl/min showed on average 15X improvement in signal-to-noise (S/N) ratio when compared to analytical flow LC for the top three major peptide catabolites detected after 60 min of incubation in rat plasma. This improvement provides additional sensitivity often required to monitor peptide catabolism in small animal studies, data shown in Figure 4-6.

Figure 2. The MetabolitePilot™ 2.0 Advanced biotransformation solution workflow to monitor degradation and catabolism products of therapeutic peptides. The therapeutic peptide sequence, any amino acid modification and list of potential amino acid biotransformations are used as an input for series of accurate mass predicted and generic LC/MS peak finding strategies to identify peptide and peptide catabolites in either IDA or SWATH® acquired LC-MS/MS data.

The Extracted Ion Chromatogram (XIC) for these three peptides shows narrow peak widths and separation of these peptides with ≥8 MS/MS points across the peptide peaks by both analytical and microflow LC-MS/MS (Figure 3).

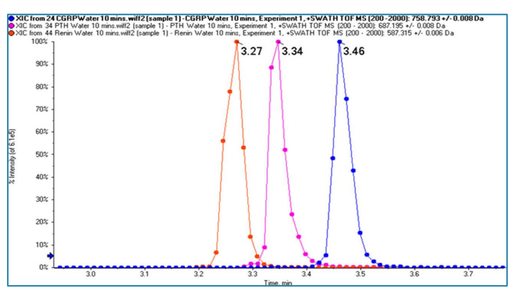

Figure 3. LC-MS XIC profile from the SWATH® acquisition of the control samples of renin substrate tetradecapeptide (orange), parathyroid hormone (pink) and calcitonin gene related peptide (blue) shows baseline chromatographic separation for these therapeutic peptides.

Peptide Catabolites Coverage

The top three catabolites for each of the therapeutic peptides identified[1] were monitored by both analytical and microflow LC-MS/MS. The m/z of the intact peptides and the catabolites monitored and detected are listed in Table 2.

Name	Formula	Monoisotopic	*m/z Observed*	Matrix
Renin Substrate Tetradecapeptide				
DRVYIHPFHLLVYS	C85H123N21O20	1759.01	879.56 (+2), 587.315 (+3)	H20
VYIHPFHLLVYS	C75H106N16O16	1486.79	496.6051 (+3)	Rat Plasma
RVYIHPFHLLVYS	C81H118N20O17	1642.89	548.6381 (+3)	Rat Plasma
DRVYIHPFHLLVY	C82H118N20O18	1670.89	557.9700 (+3)	Rat Plasma
Calcitonin Gene Related Peptide				
AC[1*]DTATC[1*]VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF[Am]	C163H267N51O49S2	3789.31	947.7425 (+4), 758.3954 (+5)	H20
SGGVVKNNFVPTNVGSKAF-[Am]	C86H137N25O25	1920.01	641.0112(+3)	Rat Plasma
AC[*1]DTATC[*1]VTHRLAGLLSR	C77H132N26O25S2	1884.92	472.2379 (+4), 629.3137 (+3)	Rat Plasma
GLLSRSGGVVKNNFVPTNVG	C88H147N27O27	2014.11	504.5336 (+4), 672.3749 (+3)	Rat Plasma
Parathyroid hormone				
SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF	C181H291N55O51S2	4117.72	686.8624 (+6), 824.0334 (+5)	H20
SVSEIQLMHNLGK	C62H106N18O20S	1454.75	485.9246 (+3)	Rat Plasma
HLNSMERVEWLRKKLQDVHNF	C119H187N37O32S	2678.38	536.6837 (+5)	Rat Plasma
LQDVHNF	C39H57N11O12	871.42	436.7164 (+2)	Rat Plasma

Table 2. The therapeutic peptides and their top three catabolism products identified. The XIC of these catabolites were monitored to check sensitivity gain by microflow LC-MS/MS.

Improved Signal to Noise Ratio (S/N)

The S/N ratio for all detected catabolites improved by average 15X with microflow LC (Figure 4,5). This resulted in better quality detection of these peptide catabolites at lower levels when compared to analytical flow LC-MRM method (Figure 6).

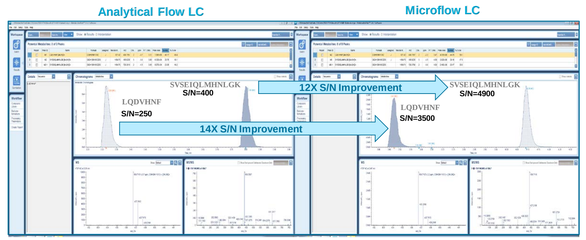

Figure 4. Microflow LC S/N Improvement. The extracted Ion chromatogram (XIC) in MetabolitePilot™ for two peptide catabolites of human parathyroid hormone fragment 1–34, shows improved S/N ratio with microflow LC as compared to data obtained by analytical flow LC.

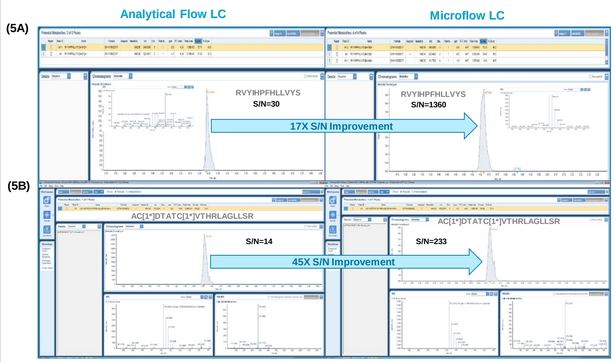

Figure 5. Microflow LC Improved Sensitivity. The MetabolitePilot™ extracted Ion chromatogram (XIC) for Renin peptide fragment RVYIHPFHLLVYS (Figure 5A) and CGRP peptide fragment AC[1*]DTATC[1*]VTHRLAGLLSR (Figure 5B), shows improved S/N ratio with microflow LC as compared to analytical flow LC.

Improved Catabolite Coverage

The improved S/N ratio with microflow LC resulted in detection of a greater number of (low concentration) peptide catabolites when compared to analytical flow LC-MS method (Figure 6).

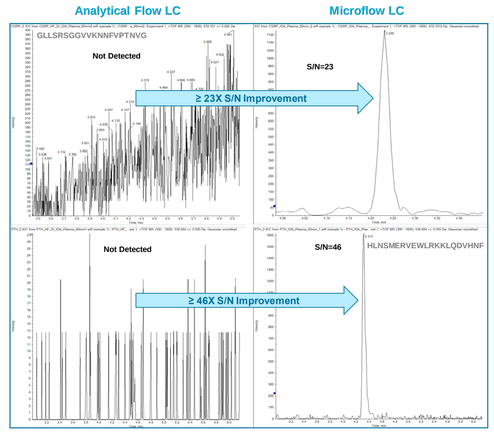

Figure 6. Microflow LC Improved Sensitivity. The PeakView® extracted Ion chromatogram (XIC) shows improved sensitivity of microflow LC-MS vs. the analytical flow LC-MS for calcitonin gene related peptide fragment GLLSRSGGVVKNNFVPTNVG, (Figure 6A) and parathyroid hormone peptide cleaved fragment , HLNSMERVEWLRKKLQDVHNF (Figure 6B), shows that the microflow LC-MS identifies these peptide fragments which are not detected in the analytical flow LC-MS data.

Conclusions

- A fast, robust, and reliable method, for the monitoring peptide catabolism in plasma.
- Signal-to-noise ratio improvement of up to 46X enables detection of catabolism products in volume limited samples and for low abundance species.
- Microflow LC method coupled to the high resolution TripleTOF® 6600 system provides optimal sensitivity and confidence to identify the low-level metabolites in complex samples when sample volume is limiting factor.

References

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