Facilitating the identification of peptide catabolites using electron-activated dissociation (EAD) through advanced software analysis

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Therapeutic peptides are a steadily expanding class of pharmaceutical agents, with more than 80 peptides approved by the FDA and many more in clinical trials.[1] Distinct from small molecules and large proteins, peptide scaffolds can offer unique advantages. Compared to small molecules, peptides can offer a lower risk of toxicity, superior affinity, specificity and limited drug-drug interaction potential.[1,2] Therapeutic peptides can be attractive due to their low immunogenicity potential compared to therapeutic proteins.[2] However, in vivo stability remains one of the critical challenges for the development of these molecules. In vitro models are widely utilized to gain insight into metabolism and understand physiological conditions. This stage is critical in the analysis of discovering and developing therapeutic peptides.

This technical note demonstrates a software-aided, streamlined approach for peptide catabolite identification using orthogonal fragmentation with the ZenoTOF 7600 system and Molecule Profiler software (**Figure 1**).

Key benefits for analysis of peptide catabolites using the ZenoTOF 7600 system with Molecule Profiler software

- **Comprehensive catabolite identification:** Achieve comprehensive profiling of peptide catabolites from plasma incubations using Zeno EAD data dependant acquisition (DDA) data generated by the ZenoTOF 7600 system
- **Site-specific identification:** Utilize a dedicated peptide processing workflow to identify proteolytic catabolites, biotransformations or a combination of both that may impact the stability of the peptide therapeutics
- **Integrated correlation function:** Compare catabolites across various samples in time course studies or inter-species comparisons using both MS/MS and analog data
- **Streamlined data processing**: Develop confident metabolic stability analyses for drug products with a quick, easy-to-use methodology from acquisition to analysis using SCIEX OS software (integrated with Molecule Profiler software)

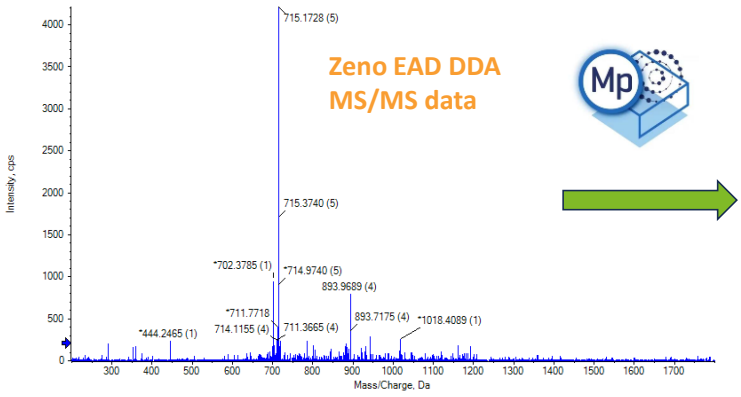

Figure 1: Comprehensive characterization and identification of peptide catabolites. Achieve comprehensive characterization and identification of therapeutic peptide catabolites using Zeno EAD DDA MS/MS data on the ZenoTOF 7600 system.

Introduction

Identifying low-level peptide catabolites poses a significant challenge due to poor MS/MS sensitivity on most accurate mass platforms. This challenge arises from the usual TOF duty cycle of 5-25%, which results in losing up to 95% of detectable ions with each scan in the MS region. However, the ZenoTOF 7600 system, equipped with the Zeno trap, addresses this issue by providing up to a 20-fold sensitivity gain without compromising speed, resolution, mass accuracy, and dynamic range.[3] The Zeno trap accumulates ions before pulsing into the TOF, increasing ion transmission to the TOF accelerator. This improvement results in an enhanced duty cycle of ≥90% across the entire mass range while performing MS/MS.[3] By unlocking higher sensitivity levels, the system can detect and characterize low-abundance catabolites that would otherwise be undetectable.

In addition to the sensitivity gains, the ZenoTOF 7600 system offers an alternative fragmentation method called electron activated dissociation (EAD). Unlike CID, which relies on ions colliding with nitrogen gas to fragment molecules, EAD involves capturing electrons by molecular ions to form a radical state that dissociates. CID and EAD produce different fragments for the same peptide, with CID yielding b and y ions and EAD yielding additional c' and z• ions. EAD fragmentation can provide complementary sequence information to CID and has the added advantage of preserving post-translational modifications that undergo neutral loss in CID experiments.[4,5]

This technical note shows that Zeno EAD DDA and Molecule Profiler software can confidently be used to identify peptide catabolites confidently.

Methods

Sample preparation: Calcitonin gene-related peptide (CGRP) was incubated at 37°C in rat plasma at a final concentration of 50 µg/mL. A 50 µL aliquot was removed from incubation and was precipitated by adding 100 µL of 1:1 (v/v) acetonitrile/methanol mixture containing 0.1% formic acid at 5, 30, 60, and 180 minute intervals.[6] The mixture was vortexed for 1 min, followed by centrifugation at 13000 rpm for 10 min at room temperature. The resulting supernatant was diluted 1:4 (v/v) with water for analysis.

Chromatography: Sample separation was performed using an ExionLC system at a 0.4 mL/min flow rate on a Phenomenex Kinetex C18 (2.1 x 100 mm, 3 µm, 100 Å) column. A 15-minute gradient was run using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B (**Table 1**). The column temperature was maintained at 40°C. An injection volume of 5 µL was used for analysis. A mixture of 1:1:1 (v/v/v) acetonitrile/methanol/water was used as a needle wash solvent.

Table 1: Chromatographic gradient used for CGRP.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	95	5
1.5	95	5
9.5	40	60
10.0	5	95
12.0	5	95
12.5	95	5
15.0	95	5

Mass spectrometry: The optimized Zeno EAD DDA parameters are listed in **Table 2** and the source and gas parameters are included in **Table 3**.

Table 2: Zeno EAD DDA parameters.

Parameter	Setting
TOF MS start–stop mass	m/z 300–2000
Maximum candidate ions	6
Accumulation time (TOF MS)	0.2 s
TOF MS/MS start–stop mass	m/z 200–1800
Accumulation time (TOF MS/MS)	0.1 s
Electron KE (EAD)	7 eV
Reaction time (EAD)	20 ms
Electron beam current (EAD)	2000 nA

Table 3: Source and gas parameters.

Parameter	Settings
Polarity	Positive
Ion source gas 1	50 psi
Ion source gas 2	50 psi
Curtain gas	35 psi
Source temperature	450°C
Ion spray voltage	5500 V
CAD gas	7

Data processing: Molecule Profiler software in SCIEX OS software, version 1.3.2, was used to identify and confirm catabolites observed in the EAD DDA data.

Identification of peptide catabolites

The Zeno trap in the ZenoTOF 7600 system significantly boosts the sensitivity of MS/MS fragments. Therefore, Zeno DDA data enables high-sensitivity EAD MS/MS spectra quality for TOF MS peaks of interest. For data processing, Molecule Profiler software can predict potential catabolites resulting from proteolytic degradation of the parent sequence as well as catabolites with putative biotransformation or a combination of both. This provides a comprehensive search space for accurate data processing. The software enables catabolite identification and confirmation by predicting putative sequences and ranking matches by leveraging EAD MS/MS fragments. Molecule Profiler software aggregates the information from multiple charge states together and provides a summary of the relative abundances and cumulative sequence coverage for the identified catabolites, facilitating rapid data reviewing.

The CGRP (AC[*1]DTATC[*1]VTH RLAGLLSRSG GVVKNNFVPT NVGSKAF-[NH2]) was used as an experimental model to demonstrate catabolite identification using Molecule Profiler software. The peptide contains a disulfide bridge and the Cys residues that form the bond are denoted by [*1] in the sequence. The precursor and fragment identification of catabolite were performed with criteria of <10 ppm mass error. Fragment ions with a signal-to-noise ratio (S/N) ≥10 or

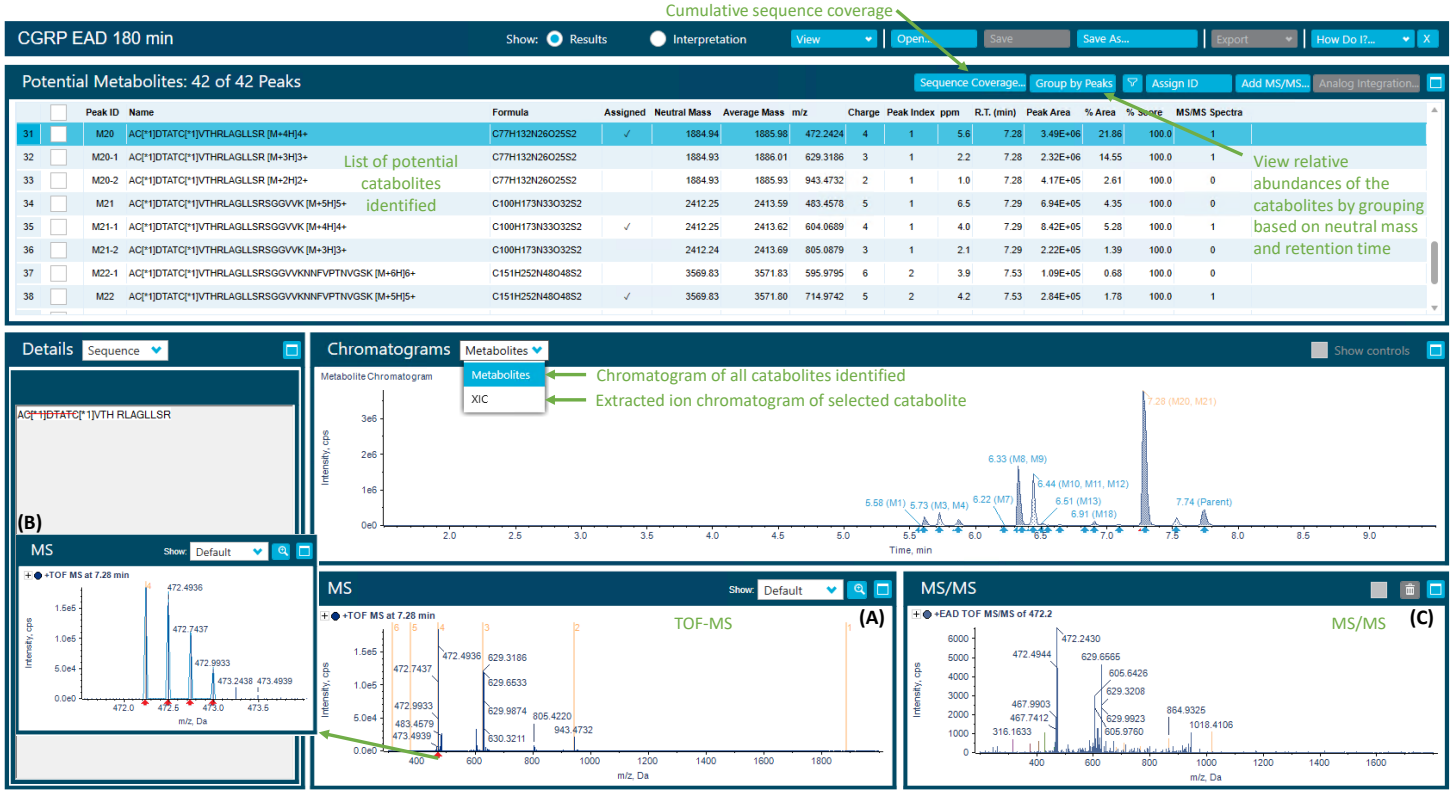

Figure 2: Results view in Molecule Profiler software. The software shows the number of potential catabolites with options to view the XIC of selected catabolite and the associated TOF MS and EAD MS/MS spectra. The TOF-MS spectrum displays the full acquired range with hyper-mass bars (shown in orange), which can be used as a quick guide for confirmation of additional charge states observed (A). A zoomed-in view of the TOF-MS spectrum of the selected charge state with a theoretical isotope pattern overlaid with experimental data is also represented (B). The red arrows indicate the isotopes used for peak area determination and are user-configurable. The MS/MS spectrum, which can be used for sequence confirmation, is also displayed (C).

higher were selected for peptide sequence assignment to minimize false positives.

The CGRP peptide incubated in plasma for 180 minutes yielded the highest number of catabolites among the 4-time points analyzed, with 22 unique catabolites identified. For each identified catabolite, the software presents an extracted ion chromatogram (XIC), TOF-MS spectrum, and MS/MS spectrum when available (**Figure 2**). The TOF-MS graph overlays the theoretical isotope pattern with the experimental data, which can be used as a guide during data review (**Figure 2B**).

The peak area for each catabolite is determined by summing XICs from multiple isotopes, which eliminates bias from overrepresenting catabolites with low masses. An alternative data perspective is the "Group by Peaks" view, which provides a summary of the relative abundances of the catabolites by combining information across multiple charge states (commonly observed for peptide catabolites) (**Figure 3**).

	Name	Neutral Mass	ppm	R.T. (min)	Peak Area	% Area
1	RLAGLLS	728.46	1.6	5.86	7.44E+04	0.47
2	NFVPTNVGSK	1061.56	4.6	5.58	9.35E+03	0.06
3	LAGLLSRSGGVV	1127.67	5.0	6.64	8.42E+03	0.05
4	NNFVPTNVGSK	1175.60	3.2	5.61	3.89E+05	2.44
5	LAGLLSRSGGVVK	1255.77	6.3	5.87	2.55E+05	1.60
6	NFVPTNVGSKAF-[NH2]	1278.67	0.8	6.44	1.11E+04	0.07
7	NNFVPTNVGSKAF-[NH2]	1392.72	3.3	6.44	1.93E+06	12.13
8	NNFVPTNVGSKAF-[NH2]+Deamidation	1393.70	2.8	6.84	1.49E+04	0.09
9	NNFVPTNVGSKAF-[NH2]+Deamidation	1393.71	4.6	6.56	2.15E+04	0.13
10	SGGVVKNNFVPTNV	1430.76	3.7	6.36	7.24E+03	0.05
11	SGGVVKNNFVPTNVG	1487.78	3.5	6.22	1.14E+04	0.07
12	AC[*1]DTATC[*1]VTHRLAGL	1528.72	4.9	6.64	3.21E+04	0.20
13	SGGVVKNNFVPTNVGSK	1702.91	3.9	5.73	4.80E+05	3.01
14	AC[*1] / DTATC[*1]VTHRLAGLLS	1746.83	-4.9	5.73	2.12E+04	0.13
15	C[*1]DTATC[*1]VTHRLAGLLSR	1813.90	3.0	7.09	5.12E+04	0.32
16	AC[*1]DTATC[*1]VTHRLAGLLSR	1884.94	5.6	7.28	6.22E+06	39.02
17	SGGVVKNNFVPTNVGSKAF-[NH2]	1920.03	3.5	6.33	2.64E+06	16.58
18	SGGVVKNNFVPTNVGSKAF-[NH2]+Deamidation	1921.02	6.4	6.42	8.17E+04	0.51
19	AC[*1]DTATC[*1]VTHRLAGLLSRSGGVVK	2412.25	4.0	7.29	1.76E+06	11.02
20	LAGLLSRSGGVVKNNFVPTNVGSK	2413.35	4.1	6.51	1.08E+05	0.68
21	LAGLLSRSGGVVKNNFVPTNVGSKAF-[NH2]	2630.47	4.2	6.91	2.04E+05	1.28
22	AC[*1]DTATC[*1]VTHRLAGLLSRSGGVVKNNFVPTNVGSK	3569.83	4.2	7.53	4.53E+05	2.84
23	Parent	3786.95	2.9	7.74	1.15E+06	7.23

Figure 3: "Group by Peaks" view. The software provides the relative abundance of identified catabolites by combining information from multiple charge states into a single table for easy review.

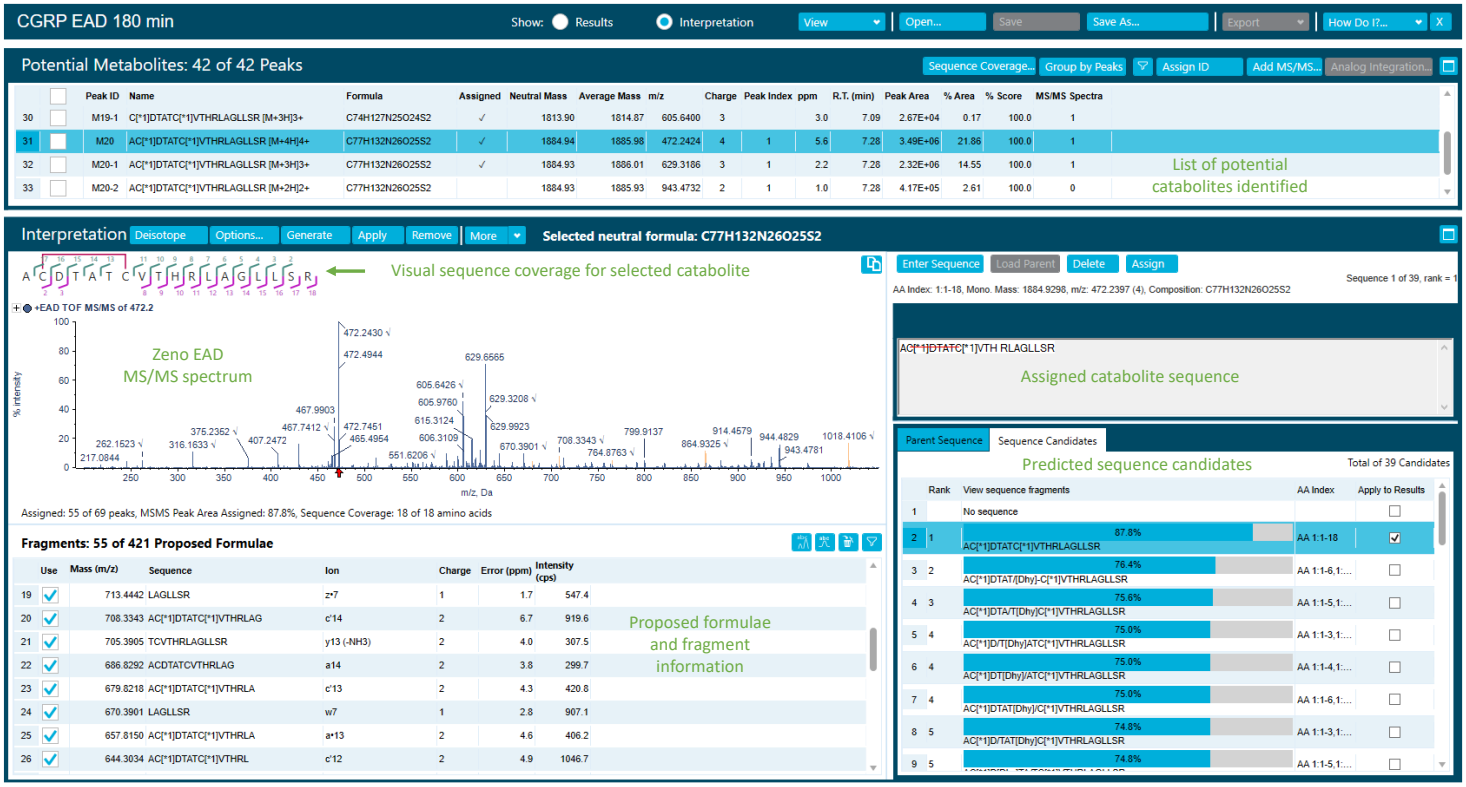

Figure 4: Interpretation view for catabolite AC[*1]DTATC[*1VTHRLAGLLSR. The Zeno EAD MS/MS spectra for the catabolite AC[*1]DTATC[*1VTHRLAGLLSR (m/z 427.2424). This sequence was ranked 1 among 38 other possible isobaric sequences based on the MS/MS spectra. The identified MS/MS fragments and their contribution are displayed as a visual sequence coverage.

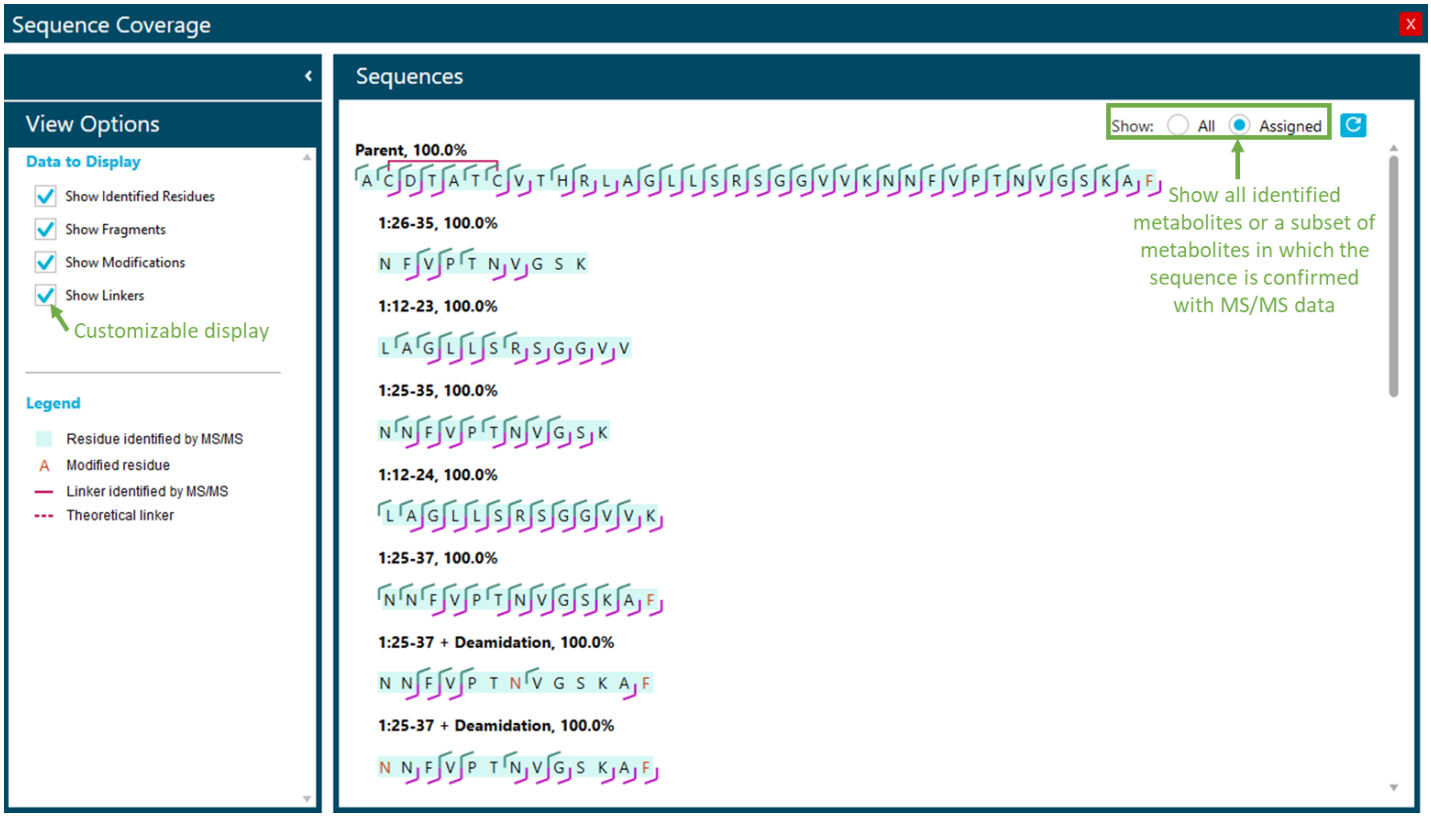

Figure 5: Sequence coverage pane of the identified catabolites. Overview of all the identified and assigned catabolites with sequence confirmation from MS/MS data.

Proteolytic catabolite AC[*1]DTATC[*1VTHRLAGLLSR, eluting at 7.28 min was identified as the most abundant catabolite in the 180 min incubation sample. Analysis of the corresponding MS/MS spectrum associated with the most abundant (+4) charge state indicated good sequence coverage obtained via EAD MS/MS data (**Figure 4**). When annotating MS/MS peaks, the software sums the intensities from all the isotopes within the charge envelope before assignment. Summation enhances the identification of low abundant multiply charged fragments without lowering the signal-to-noise (S/N) ratio, thereby minimizing false positive results.

The software provides a visual sequence coverage map for the select catabolite to determine how well the annotated MS/MS peaks match against the assigned catabolite sequence (**Figure 4**). A total of 39 isobaric species were identified as putative matches to the m/z peak of 427.2424, where the AC[*1]DTATC[*1VTHRLAGLLSR sequence was ranked 1 by Molecule Profiler software. Each proposed sequence's MS/MS evidence is represented as a percentage. The calculated percentage is based on the annotated peak area compared to the total peak area. The peak area used for the calculation is set to meet the S/N threshold for annotation.

The sequence coverage pane provides a view of the identified catabolites and the associated MS/MS for each species, enabling quick data review (**Figure 5**). The view can be customized based on preference and/or need and the catabolites shown can be easily filtered. The value for sequence coverage is cumulative, combining the MS/MS from multiple charge states.

Correlation of multiple samples

The correlation workspace of Molecule Profiler software was used to determine the presence of catabolites across several time points.

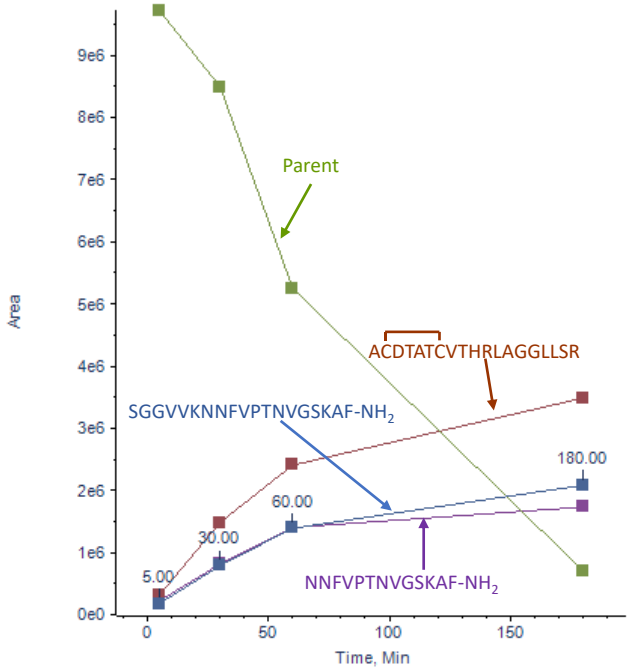

Figure 6: Correlation analysis using Molecule Profiler software. The correlation of different time points analyzed showed an increase in catabolite concentration, which was accompanied by a decrease in the concentration of the parent peptide in the incubated samples.

The correlation workspace has an interactive graph window where results can be displayed as an x-y plot, bar graph or table. In addition to the correlation workspace, overlaid XICs, TOF MS and TOF MS/MS spectra can be displayed for the chosen catabolites. **Figure 6** shows the correlation plot of the CGRP peptide and 3 of the most abundant catabolites found in all 4 different time points assessed.

A wide variety of biological matrices are utilized to study the metabolism of peptide drugs, exemplifying the complexity of these molecules, and no single organ can act as an appropriate *in vitro* system to predict the in vivo metabolism of peptide therapeutics.[2] The wide range of matrices used in metabolite identification studies is also consistent with the ubiquitous presence of peptidases in the body, where plasma and hepatic models are used more frequently.[2] This study demonstrates the ability to confidently identify therapeutic peptide catabolites formed in plasma in a streamlined manner using EAD on the ZenoTOF 7600 system coupled with Molecule Profiler software.

Conclusions

- Characterization of therapeutic peptide catabolites from rat plasma of CGRP peptide was demonstrated using Zeno EAD DDA MS/MS spectra from the ZenoTOF 7600 system
- Several catabolites from the CGRP peptide were identified and characterized using Molecule Profiler software
- A sensitive workflow enables the detection of low-level catabolites and can be easily adapted for in vivo catabolism studies with the enhanced sensitivity provided by the Zeno trap
- Diagnostic fragment ions from the Zeno EAD DDA experiment were used to identify the site of catabolism for several catabolites
- A streamlined data processing method was utilized to ease data reduction and develop confident structure-metabolic stability relationships for drugs

References

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