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Unraveling sequences of impurities and degradants of non-linear therapeutic peptides using a high-resolution LC-MS/MS workflow with electron activated dissociation

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ABSTRACT

Here, we illustrate the utility of high-resolution LC-MS/MS workflows using the electron activated dissociation (EAD) fragmentation mechanism to propose and confirm sequences of non-linear therapeutic peptides, including purely cyclic amino acid structures and peptides with chain connections through various side-chain linkers.

INTRODUCTION

Protein- and peptide-based biopharmaceuticals are emerging therapeutic candidates in biopharmaceutical development. Namely, cyclic peptides have become attractive candidate modalities since they combine several favorable properties, such as good binding affinity, target selectivity and low toxicity[1]. In order to progress through development and clinical pipelines and meet regulatory requirements, a series of pharmacokinetic properties of these candidate molecules must be optimized and well understood.

EAD was found to yield diagnostic linear peptide fragments allowing us to distinguish isomer amino-acid residues, providing superior evidence for peptide sequence confirmation and characterization of any catabolism or degradation products[2].

To provide accurate qualitative and quantitative results, dedicated data reduction tools focusing on the degradants of non-linear peptides and their radical-driven fragmentation, need to be deployed in the analytical pipeline.

MATERIALS AND METHODS

Series of peptides, Cyclosporin A, Bacitracin A, bremelanotide, CGRP, oxytocin, conotoxin and cilengitide were obtained from MilliporeSigma (MA, USA). The first 2 peptides were studied with dedicated ESI/MS/MS acquisitions (infusion). The 5 latter peptides were incubated in rat plasma at 37° C at a concentration of 50 µg/mL. At indicated time points (5, 30, 60 and 180 minutes), 50 µL aliquots were removed and precipitated with 100 µL of 1:1 (v/v) acetonitrile/methanol with 0.1% formic acid. The mixture was vortexed for 1 minute and centrifuged at maximum g for 10 minutes before 40 µL of the supernatant was diluted with 160 µL of water.

LC separation was performed on a Phenomenex Kinetex C18 column using a 5 µL injection volume. Peptides were eluted with a linear gradient using water/acetonitrile with 0.1% formic acid. A flow rate of 400 µL/min was used with a column temperature of 40° C. The samples were analyzed in positive data-dependent mode (DDA) using the ZenoTOF 7600 system (Table1).

	Sample introduction method	Infusion	LC/MS
Source and Gas parameters	Spray voltage (V)	5500	5500
	Spray Temp. (° C)	100	450
	Ion Source Gas 1 (psi)	25	50
	Ion Source Gas 2 (psi)	25	50
	Curtain Gas (psi)	35	35
	CAD Gas	7	7
	Declustering Potential (V)	100	80
	QJET RF Amplitude (V)	166	221
MS Range	MS	N/A	300-2000
	MS/MS	100-1300	100-1800
MS/MS parameters	MS/MS Accumulation time	50ms	30ms (EAD), 100ms(CID)
	CE (V)	10	dynamic
	CE (±)	6	7
	Zeno Trap	On	On
	Q1 Window	1	1

Table 1. Mass spectrometry settings for DDA with CID and EAD fragmentations.

For both the CID and EAD acquisition methods, six precursor candidates were selected within each acquisition cycle and underwent MS/MS fragmentation. The exclusion of the prior precursor candidates was based on their neutral masses, not their m/z values.

RESULTS

We compared bacitracin MS/MS datasets collected at 6 eV and 12 eV kinetic energy (KE) values. The lower KE value provided more sequence fragments (56% of MS/MS total ion current, > 500 fragments) than the higher value (34% MS/MS total ion current, < 300 fragments). We found that the studied peptides gave informative MS/MS for one or two precursor charge states at a KE value of 6 eV. In addition to the MS/MS data for the protonated precursors, MS/MS data were collected for alkali metal precursors using a DDA strategy. We found that combining fragments in these complementary MS/MS datasets provided superior information about the sequence. For example, the larger multiply charged peptide fragments were more frequent for the alkali metal precursor MS/MS, maintaining the alkali metal (Figure 1).

Data processing was performed in a research prototype version of Molecule Profiler software. To predict the peptide MS/MS fragments, the sequences of the non-linear peptides were defined in terms of their amino acid (AA) residue sequences that were supplemented with linker details (Table 2). We compared the MS/MS annotation with the in-silico generated fragments from the peptide sequence and with in-silico generated fragments from the peptide structure in a *.mol format (Figure2).

The CGRP peptide incubated in rat plasma formed several metabolites, mainly hydrolytic cleavage products. Their rate of formation and disappearance is shown in Figure 4. Both CID and EAD spectra collected with the Zeno trap on gave sufficient information to annotate the sequences and distinguish isomeric sequences.

Name	Sequence	Formula	Molecular Weight	Sequence Characteristics
Oxytocin	C[*1]YIQNC[*1]PLG-[NH2]	C43H66N12O12S2	1007.189	Disulfide bond
Bremelanotide	[1Ac]-Nle[*1,(OH)-1]HFRWK[*1]	C50H68N14O10	1025.165	Unnatural amino acid. Cyclization via side chains of AA.
Cilengitide	R[*1]GDFNmv[*1,(OH)-1]	C27H40N8O7	588.657	Unnatural amino acid. N to C cyclization.
Cyclosporin E	Abu[*1] G[1Me] L[1Me] V L[1Me] A A L[1Me] L[1Me] V T[_BMe][1Me][*1,O-1H-1]	C61H109N11O12	1188.587	Unnatural amino acid N to C cyclization.
Bacitracin	IcysLEIL[*1,N]OrnIFHDN[*1,(OH)-1]	C66H103N17O16S	1422.696	Custom aminoacid, custom cyclization.
CGRP	AC[*1]DTATC[*1]VTH RLAGLLSRSG GVVKNNFVPT NVGSKAF-[NH2]	C163H267N51O49S2	3789.312	Disulfide bond
conotoxin	C[*1]KSHypGSSC[*2]SHyp TSYNC[*3]C[*1]RSC[*2]N HypYTKRC[*3]Y-[NH2]	C120H182N38O43S6	3037.351	3 disulfide links

Table 2. The list of studied non-linear peptides.

The linker definitions in the peptide sequences have a common *[*x, formula]* format, where the *x* represents the linker index, and the *formula* outlines the addition or loss from the AA residue related to the linker connection.

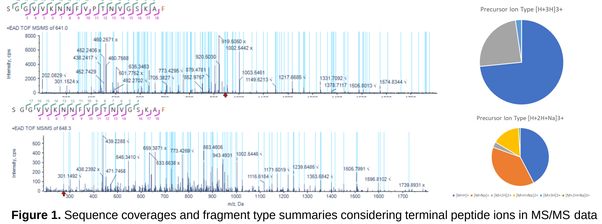


Figure 1. Sequence coverages and fragment type summaries considering terminal peptide ions in MS/MS data of the protonated and alkali metal precursors.

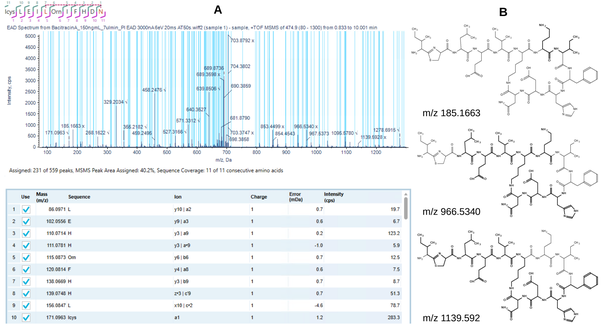


Figure 2. Bacitracin EAD MS/MS fragment annotation.

The sequence of bacitracin is outlined in Table 2. In addition to the common AA residues, the sequence contains ornithine ($C_5H_{10}N_2O$) and an isoleucyl-cysteine conjugate ($C_9H_{14}N_2OS$). The bacitracin molecule does not have a C-terminus; the asparagine is connected to the leucine through an extra amino group, creating a cycle.

By considering the expected peptide ion types (a/b/c/x/y/z) and the derived internal backbone ions, we obtained the evidence for all AA positions in the non-linear sequence and 40% of the MS/MS signal could be explained. The annotated peaks are highlighted in Figure 2A. Some fragment peaks (for example, m/z 185, 966 and 1139) could not be assigned any peptide ion types. These peaks were annotated when considering fragments resulting from up to 3 broken bonds of the bacitracin molecule structure (Figure 2B).

Figure 3. Peptide fragment annotation summary for EAD MS/MS of bacitracin $[M+3H]^{3+}$.

The bacitracin sequence contains 11 AA residues, so the number of possible terminal peptide ions is relatively small. Ion types with side-chain losses of water and ammonia were considered in addition to EAD-specific radical side-chain losses noted as *d* and *w* ion types (Figure 3).

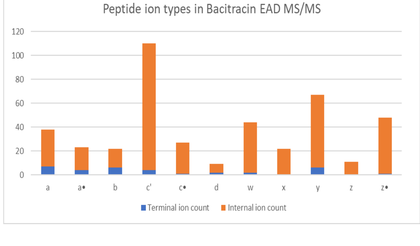
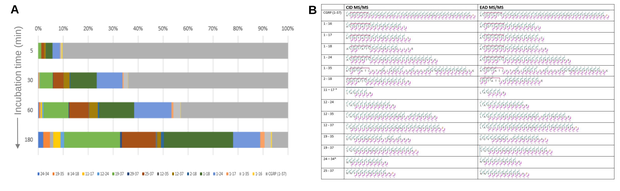


Figure 4. Results summary for the CGRP peptide incubated in rat plasma. Metabolites are named according to their position in the CGRP peptide sequence. (A) shows the change in relative abundances of CGRP peptide and metabolites with the incubation time. (B) shows the peptide sequence coverages in CID and EAD MS/MS spectra.



CONCLUSIONS

- The AA sequences of studied non-linear peptides containing both custom AA residues and custom linker chemistries allowed accurate prediction of peptide ions
- The Zeno trap activation and the DDA precursor selection enabled collection of quality MS/MS spectra, giving complete catabolite sequence coverage across 3 orders of magnitude
- Considering peptide fragments with side-chain losses, including residue-specific losses resulting in *w* and *d* fragments, aided in sequence characterization
- Integrating annotations from MS/MS spectra of different precursor ion types improved confidence in the sequence characterization
- A comprehensive annotation of the studied non-linear compounds could be achieved by combining predicted peptide fragments and fragments generated in-silico from the compound structure
- A research prototype version of the Molecule Profiler software aided in understanding the incubation products of the example non-linear peptides

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