LC-HRMS and LC-MS/HRMS Characterization of Cyclic and Bicyclic Peptides Using CID and EAD with Zeno Trapping

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BACKGROUND

Cyclic and bicyclic peptides have emerged as important drug modalities due to their improved binding affinity and stability compared to linear peptides. These properties, while advantageous for biological activity, pose analytical challenges for mass spectrometry-based structural characterization and metabolite identification. Ring opening can occur at multiple positions along the cyclic backbone, producing complex product ion spectra that hinders MS2 sequencing and interpretation. Therefore, comprehensive characterization of peptide fragmentation is essential for the accurate identification of these peptides and their metabolites during metabolism study. By Using LC–MS/HRMS in combination with CID and EAD fragmentation, we demonstrate how these complementary techniques improve confidence in structural characterization and pinpointing cleavage sites in cyclic and bicyclic peptides.

METHODS

SPSB2-iNOS inhibitory cyclic peptide-1 (CP1) and **Bicyclic UK18** incubations were performed in human, monkey, and mouse liver S9 fractions separately for 0, 0.5, 1, 3, and 3 hours at triplicates. Frozen pooled liver S9 fractions were thawed at room temperature and diluted to desired concentration using PBS buffer. The reaction mixture contained 2 mg/mL S9 protein, 3 μM of test articles, and 1 mM of NADPH. The reaction was terminated by crashing the samples at 1:3 volume ratio with ACN:FA (100:0.1, v:v) containing Warfarin as an internal standard. The crashed samples were centrifuged, and the supernatants were collected and dried under gentle N_2 stream. The samples were reconstituted with ACN:H2O:FA (5:95:0.1, v:v:v) for LC-HRMS and LC-MS/HRMS analysis.

A Shimadzu UHPLC system was coupled to a SCIEX ZenoTOF 7600 for sample analysis. Separation was performed using a Phenomenex Kinetex 2.6u EVO C18 100A (100 x 2.1 mm) column at a flow rate of 0.35 mL/min, with the column temperature set to 60 °C. Mobile phase A consisted of 0.1% FA in water and mobile phase B was 0.1% FA in acetonitrile. The gradient was run from 5% to 40% B over 8 min, followed by a hold at 98% for 2min, and then re-equilibration to 5% B. Data was acquired in positive mode with a Sequential Window Acquisition of All Theoretical (SWATH) or MRM HR method for MS/HRMS. Molecule profiler (SCIEX, Massachusetts, USA) was used to assist identification of metabolites and annotation of MS/MS fragments.

For metabolic stability, the % remaining of test articles was calculated using peak area ratio to the internal standard and taking the average peak area ratio relative to the average at 0 hours.

RESULTS

To compare the CID and EAD fragmentation, an MRM HR method was used to simultaneously acquire TOF MS, CID MS/MS, and EAD MS/MS.

For CP1, CID primarily yielded backbone b/y fragments, whereas EAD promoted disulfide bond cleavage, evidenced by detection of opened precursor species, and generated subsequent sequence-informative b/y and c/z fragments (Figure 2).

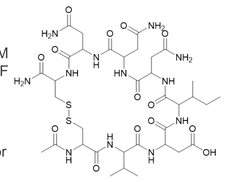

Figure 1. Structure of CP1

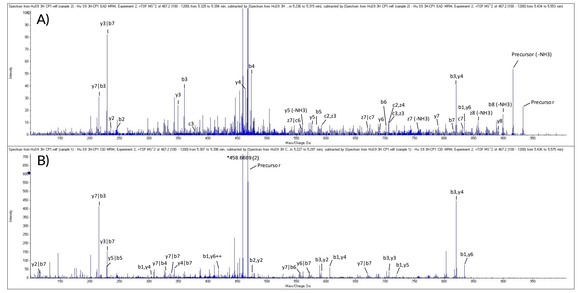

Figure 2. CP1 standard under CID vs EAD conditions. (A) EAD with a kinetic energy of 7 eV and beam current of 3000 nA. (B) CID with a collision energy of 22 eV.

- The % remaining after 24H incubation for CP1 is 1%, 5%, and 12% in human, monkey, and mouse S9, respectively (Figure 3).
- Human S9 samples were used for metabolites characterization. Incubation of CP1 generated metabolites primarily through disulfide bond reduction followed by sequential amino acid truncation from the newly formed N-terminus (Table 1).
- Most metabolites were detected predominantly as singly charged ions with CID provides structurally informative product ions enabling sequence confirmation.
- EAD produced limited fragmentation for these singly charged precursor ions, which are generally less favorable for efficient electron-based dissociation.

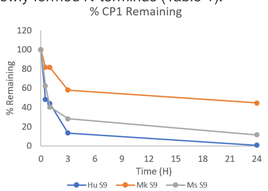

Figure 3. The % remaining CP1 over the 24H incubation in human, monkey, and mouse liver S9.

Table 1. Summary table of CP1 metabolites in human S9 incubation

Name	RT	Chemical Formula	m/z	Charge	Monoisotopic Mass	Sequence
Parent	4.61	$C_{35}H_{56}N_{12}O_{14}S_2$	467.1813	2	932.348	Ac-C[*1]VDINNNC[*1]-Ami
M1	4.68	$C_{35}H_{58}N_{12}O_{14}S_2$	468.1891	2	934.3637	Ac-CVDINNNC-Ami
M2	2.40	$C_{30}H_{51}N_{11}O_{12}S$	790.3512	1	789.3439	VDINNNC-Ami
M3	1.00	$C_{25}H_{46}N_{10}O_{11}S$	691.2828	1	690.2755	DINNNC-Ami

Similarly, CID and EAD fragmentation were compared for bicyclic UK18. CID fragmentation was dominated by internal b/y-type ions arising primarily from cleavages with loop 1 and loop 2. Fragmentation near the TBMB linkage was comparatively limited under CID conditions.

In contrast, EAD produces complimentary c/z ions and also generated a number of fragments associated with cleavage adjacent to the central scaffold (Figure 5).

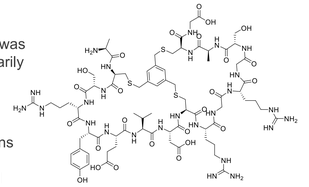

Figure 4. Structure of UK18

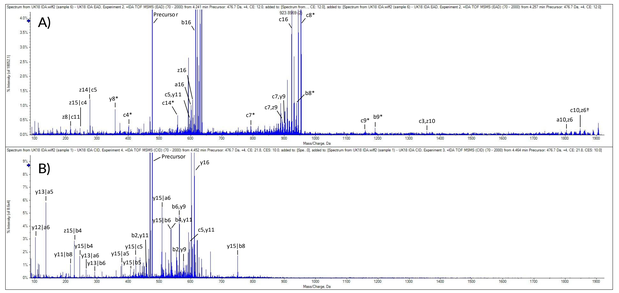

Figure 5. UK18 standard under CID vs EAD conditions. (A) EAD with a kinetic energy of 7 eV and beam current of 3000 nA. (B) CID with a collision energy of 22 eV. * indicates fragments breaking at thioether linkage to the core TBMB scaffold. † indicates fragments with multiple possible structures.

- Following 3H incubation, UK18 showed 11%, 15%, and 48% remaining in human, monkey, and mouse S9 fractions, respectively. After 24 h incubation, the remaining parent compound decreased to less than 1% across all species (Figure 6).
- Human S9 fractions were selected for metabolite characterization. Incubation of UK18 in S9 generated metabolites primarily through peptide bond hydrolysis. Initial metabolism involved cleavage of the N-terminal alanine, followed by various truncation within both loop 1 and loop 2 of the bicyclic peptide scaffold.

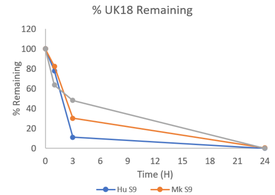

Figure 6. The % remaining UK18 over the 24H incubation in human, monkey, and mouse liver S9.

- The final metabolite, M12, consisted of cysteines remaining attached to the TBMB core scaffold. M11, corresponding to M12 with the C-terminal glycine still attached, was identified as the predominant metabolite following 24 h incubation, accounting for 52% of total peak area.
- Several metabolites, including M2, M3, and M10, were associated with multiple candidate structures based on the observed m/z values. CID and EAD MS/MS fragmentation data enabled confident structural assignment of the most plausible metabolite sequences. MS/MS spectra for M2 are shown in Figure 7 as a representative example.

Table 2. Summary table of UK18 metabolites in human S9 incubation

Name	RT	Chemical Formula	m/z	Charge	Monoisotopic Mass	Sequence
Parent	4.48	$C_{77}H_{118}N_{26}O_{26}S_3$	476.7054	4	1902.792	ACSRYEVDCRGRGSACG
M1	4.18	$C_{74}H_{113}N_{25}O_{26}S_3$	458.9461	4	1831.755	CSRYEVDCRGRGSACG
M2	3.84	$C_{74}H_{115}N_{25}O_{25}S_3$	463.4487	4	1849.766	CSR/YEVDCRGRGSACG*
M3	3.72	$C_{71}H_{113}N_{24}O_{24}S_3$	449.1939	4	1792.744	CSRYEVDCRGR/SACG*
M4	2.97	$C_{60}H_{96}N_{18}O_{20}S_3$	390.4228	4	1557.66	CSR/VDCRGRGSACG
M5	4.00	$C_{60}H_{96}N_{18}O_{20}S_3$	493.8658	3	1478.574	CSRYEVDCRG/CG
M6	3.53	$C_{51}H_{82}N_{14}O_{17}S_3$	439.1834	3	1314.527	C/VDCRGRGSACG
M7	2.84	$C_{49}H_{78}N_{14}O_{16}S_3$	435.1713	3	1302.49	CS/DCRGRGSACG
M8	3.09	$C_{46}H_{73}N_{13}O_{16}S_3$	406.1606	3	1215.458	C/DCRGRGSACG
M9	3.43	$C_{57}H_{92}N_{20}O_{20}S_3$	491.8731	3	1472.596	ACS/VDCRGRGSACG
M10	2.77	$C_{23}H_{39}N_7O_8S_3$	606.1726	1	605.1648	AC/C/CG*
M11	2.14	$C_{20}H_{35}N_7O_7S_3$	535.1355	1	534.1277	C/C/CG
M12	1.70	$C_{18}H_{27}N_7O_6S_3$	478.1140	1	477.1062	C/C/C

* Multiple candidate structures were associated with the observed *m/z* values. The most plausible sequence assignments, supported by CID and EAD MS/MS fragmentation data, are reported.

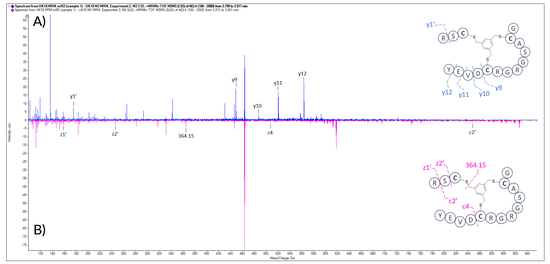

Figure 7. CID and EAD MS/MS spectra for UK18 metabolite M2 with annotation of diagnostic product ions.

CONCLUSIONS

- CID and EAD provided complementary fragmentation for cyclic and bicyclic peptides, improving structural characterization and metabolite identification.
- For cyclic peptide CP1, CID predominantly generated backbone b/y ions, while EAD promoted disulfide bond cleavage and produced additional sequence-informative fragments.
- For bicyclic UK18, CID fragmentation was dominated by internal b/y ions within loop regions, whereas EAD generated complementary c/z ions and enhanced cleavage near the TBMB thioether linkages.
- Metabolite profiling in liver S9 fractions demonstrated peptide bond hydrolysis and sequential amino acid truncation for both peptide systems.
- Combined CID and EAD MS/MS enabled confident structural assignment of metabolites with ambiguous candidate sequences, demonstrating the potential of integrating EAD and CID techniques for characterization of complex cyclic peptide therapeutics and their biotransformation pathways.