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Application Note

Characterization and Impurity Profiling of Combined Amylin and GLP-1 Analogs with RapiZyme Trypsin

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Abstract

Glucagon-like peptide 1 receptor agonists (GLP-1RAs) are a class of biotherapeutic peptide-like drugs currently undergoing a rapid increase in demand and use. These peptide-based therapeutics are often fatty acid modified to improve their potency, complicating their reversed phase (RP) impurity analysis. Combined lipopeptide therapeutics that target more than one receptor are in development for improved treatment of Type 2 diabetes and weight loss. Here, Waters™ RapiZyme™ Trypsin is used for the characterization and impurity analysis of combined amylin and GLP-1 analogs, cagrilintide and semaglutide. RapiZyme Trypsin delivers a comprehensive, bottom-up analysis of the combined therapeutic with more than 99.5% digestion completion, thereby aiding in the characterization of peptide residue modifications.

Benefits

- RapiZyme Trypsin delivers a comprehensive peptide map of combined cagrilintide and semaglutide with >99.5% digestion completion

- Digestion improves chromatographic separation of peptides with minor amino acid residue modifications
- Liquid chromatography-mass spectrometry (LC-MS) analysis of digests facilitates peak identification and characterization of peptide residue modifications

Introduction

GLP-1RAs are a class of biotherapeutic peptide-like drugs developed to treat Type 2 diabetes. The recent Food and Drug Administration (FDA) approval of multiple GLP-1RAs for weight loss has led to a rapid increase in their demand and use, prompting a need for robust chromatographic methods to screen their composition and purity.[1] These peptide-based therapeutics are often fatty acid modified to improve their potency. Fatty acid modification complicates the RP analysis of GLP-1RAs because the highly hydrophobic lipid dominates the observed retention effects, hindering the RP separation of impurities corresponding to modified and degraded amino acid residues. Consequently, shallow gradients and long run times are often required to achieve a satisfactory impurity profile of fatty acid modified GLP-1RAs.

Combined lipopeptide therapeutics that target more than one receptor are in development for Type 2 diabetes and weight loss. CagriSema® (Novo Nordisk), a drug currently in late-stage clinical trials, combines an amylin and a GLP-1 analog in a single formulation. CagriSema is a triple agonist, targeting GLP-1 receptors with semaglutide and amylin and calcitonin receptors with cagrilintide.[2] Combining peptide therapeutics further complicates reversed phase impurity analysis, as active pharmaceutical ingredient (API) and impurities of the individual drug substances may co-elute with one another. Thus, impurity analysis via proteolytic digestion may be particularly advantageous for the impurity analysis of combined therapeutics.

Proteolytic digestion is used in early-stage characterization of peptide therapeutics and as a comparative fingerprint in lot release testing. Here, RapiZyme Trypsin is used for the characterization and impurity analysis of combined amylin and GLP-1 analogs, cagrilintide and semaglutide (Figure 1). Digestion with RapiZyme Trypsin delivers a comprehensive bottom-up fingerprint analysis of the combined therapeutic with >99.5% digestion completion. Moreover, digestion improves the chromatographic separation and mass spectrometry identification of minor impurity species.

Figure 1. Structures of cagrilintide and semaglutide. Trypsin cleavage sites are shown in light blue circles and deamidation sites are shown in orange circles.

Experimental

Sample Preparation

Cagrilintide (Cayman Chemical, p/n: 41329) and semaglutide (AA Blocks, p/n: 910463-68-2) were dissolved in tris-HCl (100 mM, pH 7.5) to prepare 1 mg/mL stock solutions. 5 µL of each stock solution were added to 27.5 µL tris-HCl with 5 µL of acetonitrile and 5 µL of 1.25 mM N-ethylmaleimide (NEM). The blocking reaction proceeded for 2 hours at room temperature followed by addition of 2.5 µL of 0.1 mg/mL RapiZyme Trypsin (p/n: 186010108 < https://www.waters.com/nextgen/global/shop/standards--reagents/186010108-rapizyme-tryspin-ms-grade-4-pk.html>). The digest was incubated at 37 °C for 30 minutes and quenched with 5 µL of 1% formic acid. For digests with partial reduction, the sample was incubated with 1.25 nmol tris(2-carboxyethyl)phosphine (TCEP) for 30 minutes at room temperature prior to NEM blocking.

LC-MS Conditions

LC Conditions

LC system:	ACQUITY™ Premier UPLC™ System
Column:	ACQUITY Premier Peptide CSH™ C_{18} Column, 130 Å, 1.7 µm, 2.1 x 150 mm (p/n: 186009489)

Column temperature:	60 °C
Sample temperature:	6 °C
Injection volume:	10 µL (digests) or 1 µL (intact)
Mobile phase A:	0.1% formic acid in H_2O
Mobile phase B:	0.1% formic acid in acetonitrile
Detection λ:	214 nm
Sample vials:	QuanRecovery™ MaxPeak™ 12 x 32 mm Propylene 300 µL Screw Cap Vials (p/n: 186009186)

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.2	99	1	Initial
1	0.2	95	5	6
30	0.2	35	65	6
33	0.2	30	70	6
35	0.2	30	70	6
36	0.2	99	1	6
50	0.2	99	1	6

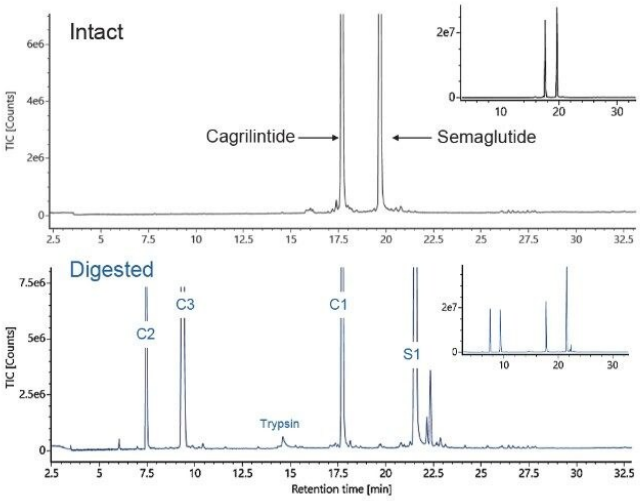
RDa Detector Settings

Mass range:	50–2000 *m/z*
Mode:	ESI+ full scan

Sample rate:	10 Hz
Capillary voltage:	1.2 kV
Cone voltage:	30 V
Desolvation temperature:	550 °C
Data management:	waters_connect™ Software

Results and Discussion

The chromatograms of intact and digested cagrilintide/semaglutide samples are shown in Figure 2. All expected tryptic peptides of cagrilintide and a single tryptic peptide of semaglutide are observed in the digested sample. The two remaining semaglutide tryptic peptides, S2 and S3, are too small to be observed with the presented LC-MS conditions. Digestion completion was calculated using the integrated extracted ion chromatograms (XICs) of all observed tryptic and undigested peptides; >99.5% digestion was achieved for both cagrilintide and semaglutide.



Peptide	Sequence	Monoisotopic mass (Da)
Cagrilintide	K(mod)CNTATCATQRLAEFLRHSSNNFGPILPPTNVGSNTP	4409.25
Semaglutide	HXEGTFTSDVSSYLEGQAAK(mod)EFIAWLVRGRG	4111.12
C1	K(mod)CNTATCATQR	1646.83
C2	LAEFLR	747.43
C3	HSSNNFGPILPPTNVGSNTP	2048.99
S1	HXEGTFTSDVSSYLEGQAAK(mod)EFIAWLVR	3840.98
S2 (not observed)	GR	231.13
S3 (not observed)	G	75.03

Figure 2. Total ion chromatograms of intact cagrilintide/semaglutide (top) and RapiZyme Trypsin-digested cagrilintide/semaglutide (bottom). Sequence and mass information for the intact and tryptic peptides are listed in the table.

Prior to digestion, cagrilintide/semaglutide samples were treated with NEM to identify free sulfhydryls and prevent disulfide scrambling during digestion. To confirm successful NEM blocking, a cagrilintide/semaglutide sample was partially reduced to generate free sulfhydryls prior to treatment with NEM. The chromatograms of the non-reduced and partially reduced digests are shown in Figure 3. A new peak at 19 minutes with a mass corresponding to C1+2NEM is observed in the partially reduced digest, confirming successful reaction between NEM and free sulfhydryls. This species is not observed in the non-reduced digest, indicating essentially complete disulfide bond formation in cagrilintide.

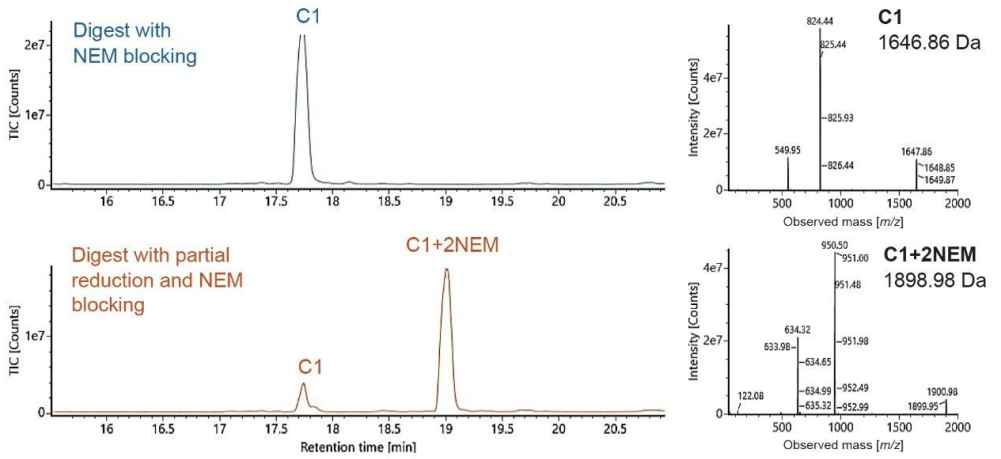


Figure 3. Total ion chromatograms of non-reduced (top) and partially reduced (bottom) cagrilintide/semaglutide digests. The mass spectra for C1 and C1+2NEM are shown on the right.

To demonstrate the benefits of RapiZyme Trypsin digestion in the impurity analysis of lipopeptides, an unknown impurity was assessed with both intact and digested cagrilintide/semaglutide samples. Figure 4 displays the zoomed chromatogram of intact cagrilintide/semaglutide. A species with a +3 Da mass shift relative to cagrilintide elutes just after unmodified cagrilintide (1103.31 *m/z*), potentially corresponding to a triply deamidated species. Elucidating its identity from intact data alone is not possible without further interrogation with MS/MS. Moreover, this species co-elutes with another impurity (1074.56 *m/z*). Trypsin digestion, on the other hand, enables deeper understanding of the modified residues within this impurity and improves the ability to monitor those modifications chromatographically.

The six possible deamidation sites of cagrilintide are localized on the C3 and C1 tryptic peptides. Figure 4 displays the zoomed chromatogram of C3 and C1. Two species with a +1 Da mass shift relative to C3 elute just after unmodified C3. This mass shift correlates to a singly deamidated C3, and the two chromatographically separated peaks are consistent with aspartic acid and isoaspartic acid deamidation isomers of C3. A tryptic peptide with a +2 Da mass shift relative to C1 elutes just after unmodified C1. NEM blocking prior to digestion confirms that this species is not reduced C1, instead suggesting that the +2 Da mass shift corresponds to a doubly deamidated C1. Notably, neither the +1 Da C3 impurity nor the +2 Da C1 impurity co-elute with neighboring tryptic peptides present in the sample.

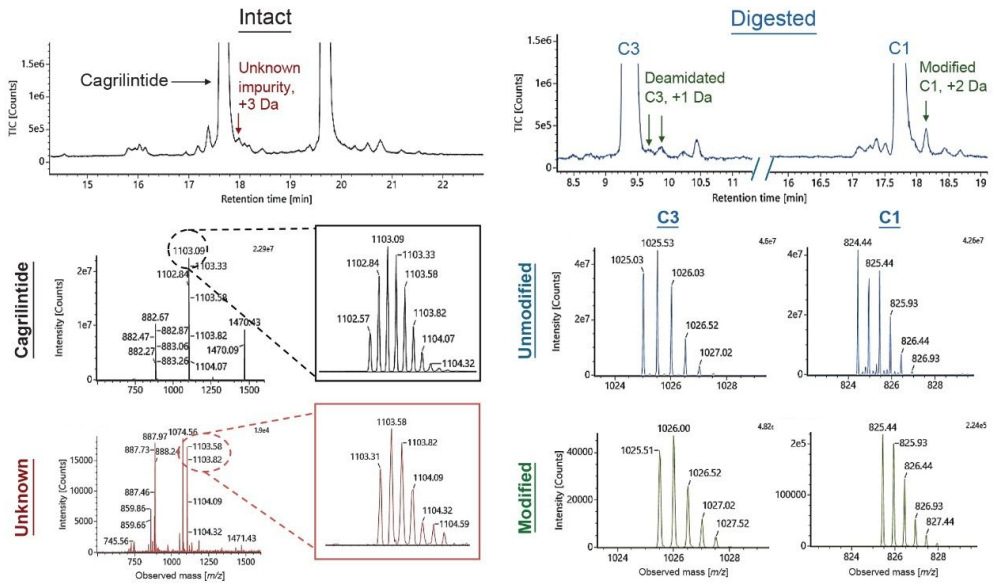


Figure 4. Zoomed TIC of intact cagrilintide/semaglutide and associated modified and unmodified cagrilintide mass spectra (left) and the zoomed TIC of cagrilintide tryptic peptides and associated modified and unmodified mass spectra (right).

To gain further insight into the identity of the unknown cagrilintide impurity, a cagrilintide/semaglutide sample was incubated at 50 °C for four days at pH 7.5 to force deamidation prior to digestion. The XICs of deamidated C3 for the fresh and degraded samples are shown in Figure 5. In the degraded sample, a series of deamidated C3 species elute just after the main C3 peak. The peaks denoted with a star have a mass consistent with singly deamidated C3 and the peaks denoted with a circle have a mass consistent with doubly deamidated C3. The retention time of two of the singly deamidated species in the degraded sample align with those of the +1 Da impurity species in the fresh sample. This data confirms the identity of the +1 Da impurity species observed in the fresh sample as singly deamidated C3.

The XICs of deamidated C1 are shown on the right in Figure 5. In the degraded sample, two deamidated C1 species elute just after the main C1 peak. The mass spectra for each of these peaks correspond to singly deamidated C1. The retention time of the first eluting peak aligns with that of the +2 Da impurity species observed in the fresh sample. The isotopic distribution of this first eluting peak (green triangle) differs from that of the second eluting peak (green square); the relative abundance of the 825.42 *m/z* ion is greater in the first eluting peak. This suggests that the +2 Da impurity species is present and co-elutes with the first eluting

deamidated C1 species in the degraded sample. From this data, the identity of the +2 Da impurity species cannot be confirmed as doubly deamidated C1 as formation of this species was not observed in forced degradation experiments. Regardless, it is clear that the unknown impurity observed in intact cagrilintide/semaglutide sample contains a deamidated site localized on the C3 peptide and an additional +2 Da modification localized on the C1 peptide.

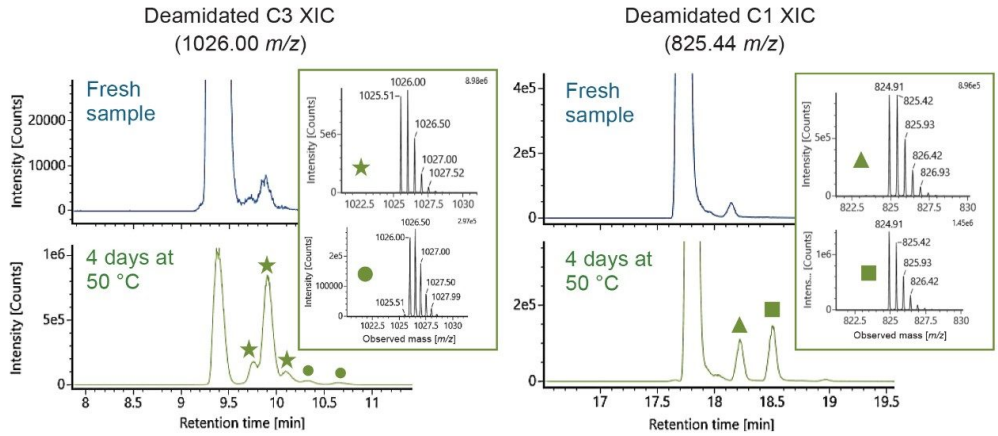


Figure 5. The XICs of deamidated C3 and C1 peptides for fresh and degraded cagrilintide/semaglutide samples. The mass spectra of the deamidated species generated during forced degradation are shown in the insets.

Conclusion

RapiZyme Trypsin was used to digest combined amylin and GLP-1 analogs, cagrilintide and semaglutide. Digestion with RapiZyme Trypsin delivers a comprehensive bottom-up fingerprint of the combined therapeutic with >99.5% digestion completion and improved chromatographic separation of peptides with minor amino acid residue modifications. Moreover, digestion with RapiZyme Trypsin enables tentative identification of unknown impurity species with localized understanding of peptide modifications.

References

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