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Unambiguous di-sulphide bond assignment in synthetic peptides Linaclotide and Plecanatide using Agilent 6546/6550 LC-QTOF High Resolution Mass Spectrometer

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Introduction

Disulphide linkages and functionality

Di-sulphide bridges are critical for secondary structure and functionality of the peptide/protein. Identification of the Cysteines involved in di-sulphide bond formation is a challenge. Close spacing of cysteines esp. in small peptides poses further challenges.

Disulphide locations in Linaclotide and Plecanatide

- Linaclotide is 14 amino-acid long peptide with 3 di-sulphide bridges involving 6 Cys residues viz.Cys1-Cys6, Cys2-Cys10 and Cys5-Cys13.
  - The sequence is CCEYCCNPACTGCY
- Plecanatide is a 16 amino acid peptide with two di-sulphide bridges between Cys4-Cys12 and Cys7-Cys15
  - The sequence of Plecanatide is NDECELCVNVACTGCL

Ensuring correct di-sulphide linkages during the synthesis of these molecules is very important as some participating Cysteines are closely located.

Experimental

Di-sulphide peptide cleavage.

The Peptide were subjected to partial reduction of di-sulphide bridges by Tris(2-carboxyethyl)phosphine hydrochloride TCEP.

The methodology used by Go´ngora-Benı´tez et al.[1] was followed for linaclotide however peptide concentration and the TCEP ratios were optimized further for Plecanatide.

The reduced peptides were Cyanylated by using CDAP and these cyanylated peptides were separated on HPLC and collected as separate fractions.

Fractionated peptides were cleaved at Cyanylated Cysteines by incubating with 1 M Ammonia and 6M Gdn-Hcl at 25°C for 25 minutes.

Mass-spectrometric analysis

All the samples were analyzed on Agilent 6550 or 6546 LC/Q-TOF platform coupled with Agilent 1290 Infinity II HPLC

The same instrument was used to monitor the partial reduction, cyanylation and cleavage steps . The sequence of the peptide fragments were confirmed by MS/MS data.

Peptide fractionation was carried out on high binding capacity column and fractions were collected manually after UV detection.

Experimental

Fig1: 6546 LC/Q-TOF with 1290 Infinity II

Results and Discussion

Di-sulphide bond confirmation in Linaclotide

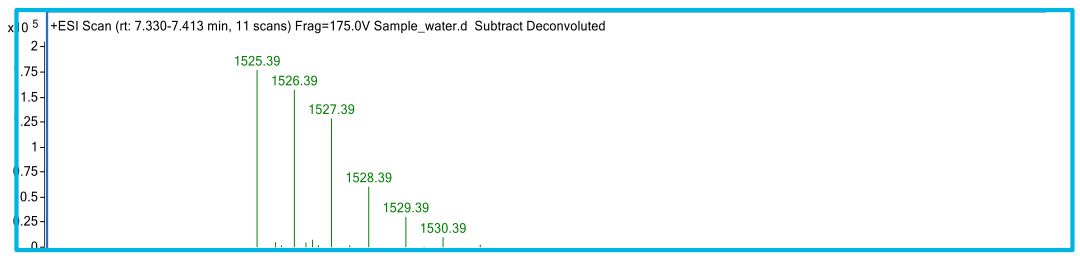

Fig 2a: Deconvoluted Mass-spectrum of native Linaclotide

Mass of native Linaclotide molecule was observed as 1525.39 Da which was found to increase by 2 Da on reduction of one di-sulphide bond to 1527.41 Da. Three distinct peaks with 1527.41, 1527.42, 1527.41 were observed respectively after reduction of the three di-sulphide bonds one at a time. After Cyanylation, the mass was found to be 1577.40 Da..

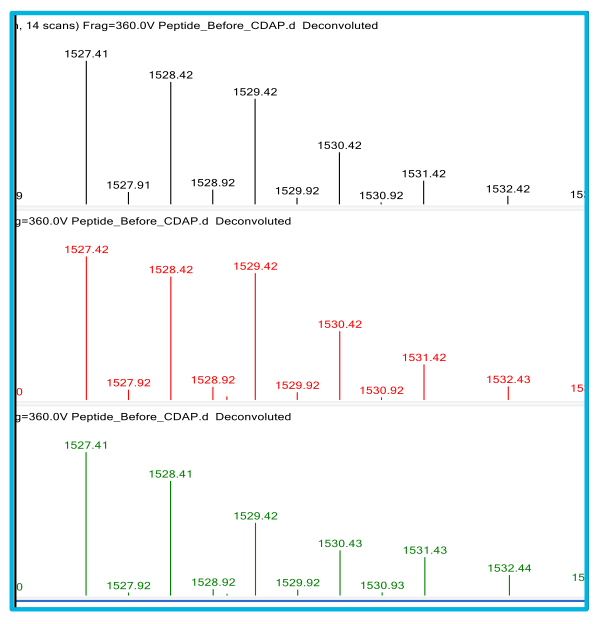

Fig 2b: Deconvoluted Mass-spectra of three reduced di-sulphide peaks.

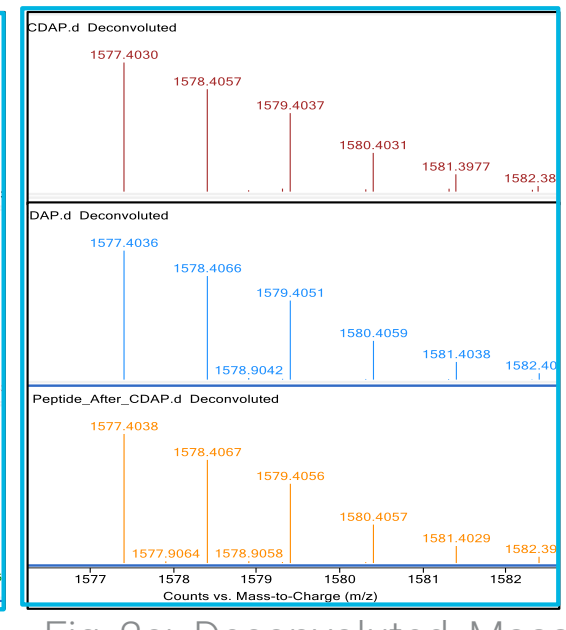

Fig 2c: Deconvoluted Mass-spectra of the peaks after CDAP treatment.

Confirmation of Cys1-Cys6 bond in Linaclotide

- Cleavage of the Cys1-Cys6 bond results in the formation of two peptide fragments of CCEY C (643.16 Da) and CNPACTGCY (955.310 Da).The sequence of both fragments is was confirmed with MS/MS pattern

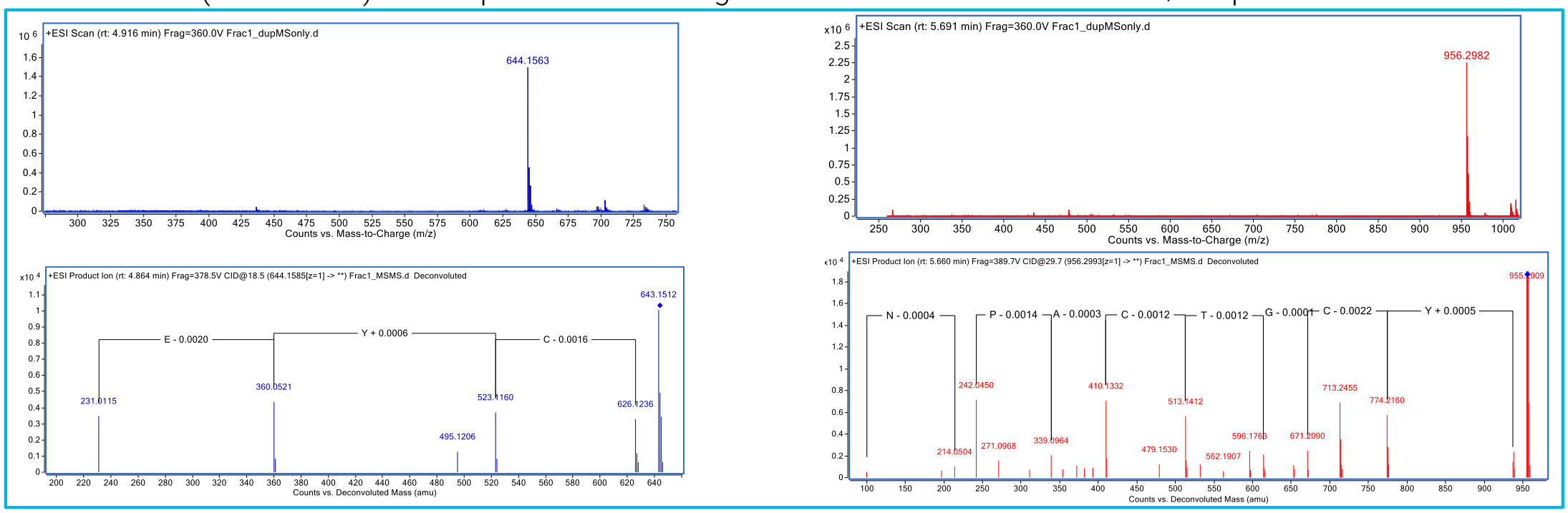

Fig 3a : MS and MS/MS spectrum of the peptide fragments (m/z 644.1563 and 956.2982)confirming the Cys1-Cys6 bond.

Confirmation of Cys2-Cys10 bond in Linaclotide

- The cleavage of Cys2-Cys10 di-sulphide bond results in three signature peptide fragments with sequence CC(120.04 Da), CTGCY(570.165 Da) and CEYCCNPA(925.29Da)

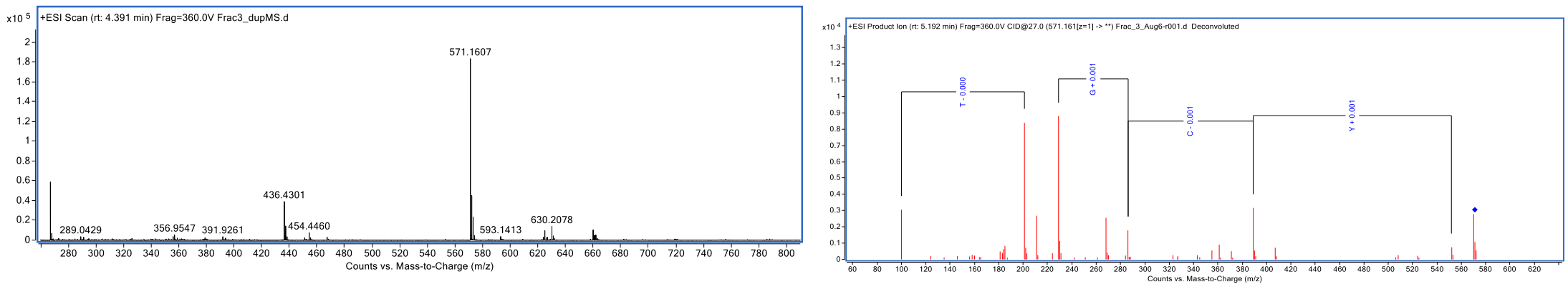

Fig 3b : MS and MS/MS spectrum of the peptide fragments m/z 571.1607 confirming the Cys2-Cys10 bond.

Confirmation of Cys5-Cys13 bond in Linaclotide

The cleavage of Cys5-Cys13 bond results in 3 fragments CCEY (516.1349 Da), CCNPACTG (791.2275 Da) and CY(309.0783 Da)

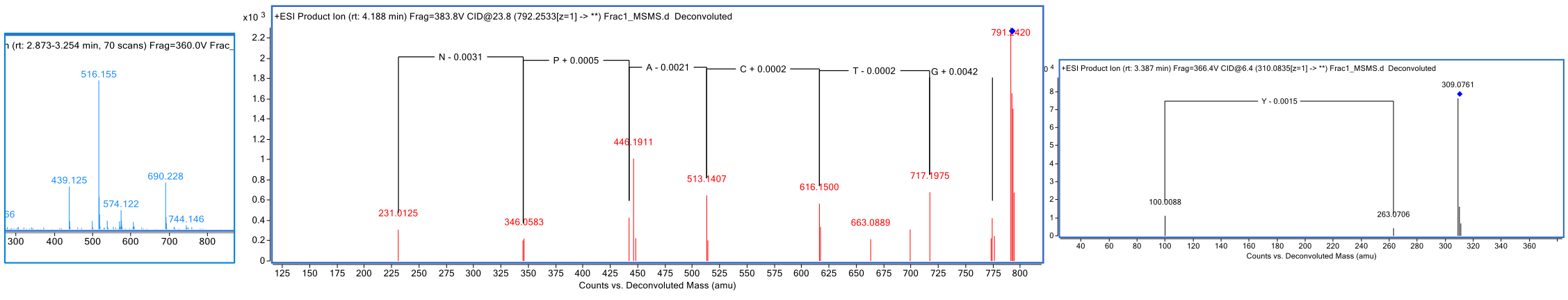

Fig 3c : Deconvoluted MS and MS/MS spectrum of the peptide fragments CCEY (516.1349 da), CCNPACTG (791.2275 da) and CY(309.0783 Da)

Results and Discussion

Di-sulphide bond confirmation in Plecanatide

The partial reduction of one of the two di-sulphide bonds in Plecanatide was obtanined by optimizing TCEP to peptide ratio and was monitored on Agilent 6546 LC/Q-ToF Mass-spectrometer.

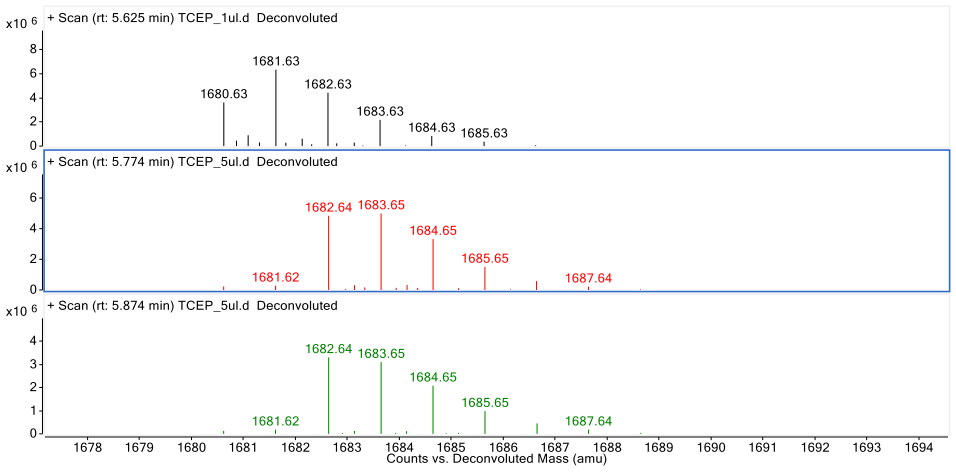

Fig 4a: Deconvoluted Mass-spectra of two separate peaks showing reduced di-sulphide linkage by increment of mass from 1680.63 Da to 1682.64 Da

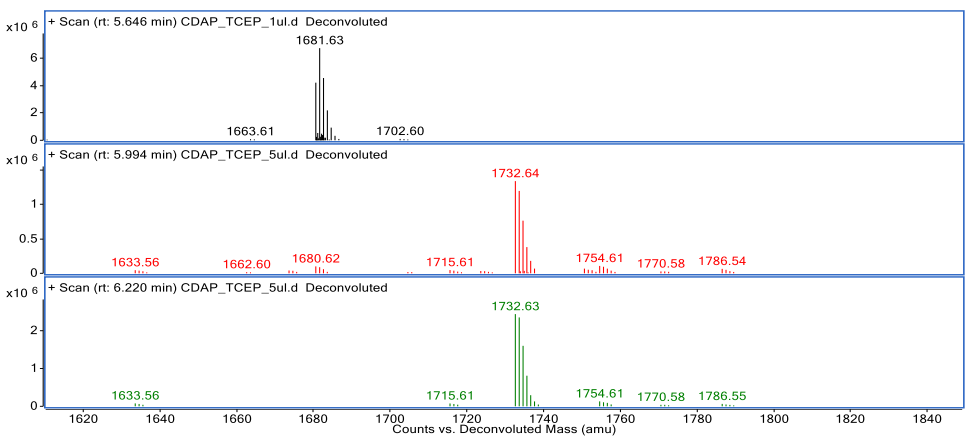

Fig 4b: Deconvoluted Mass-spectra confirming Di-Cyanylation of the reduced di-sulphide links and increasing of 50 Da by addition of two CN residues instead of H (1732.64 Da)

Confirmation of Cys7-Cys15 linkage

The cleavage of Cys7-Cys15 bond results in two fragments NDECEL (721.2589 da) and CVNVACTG (789.33 Da)

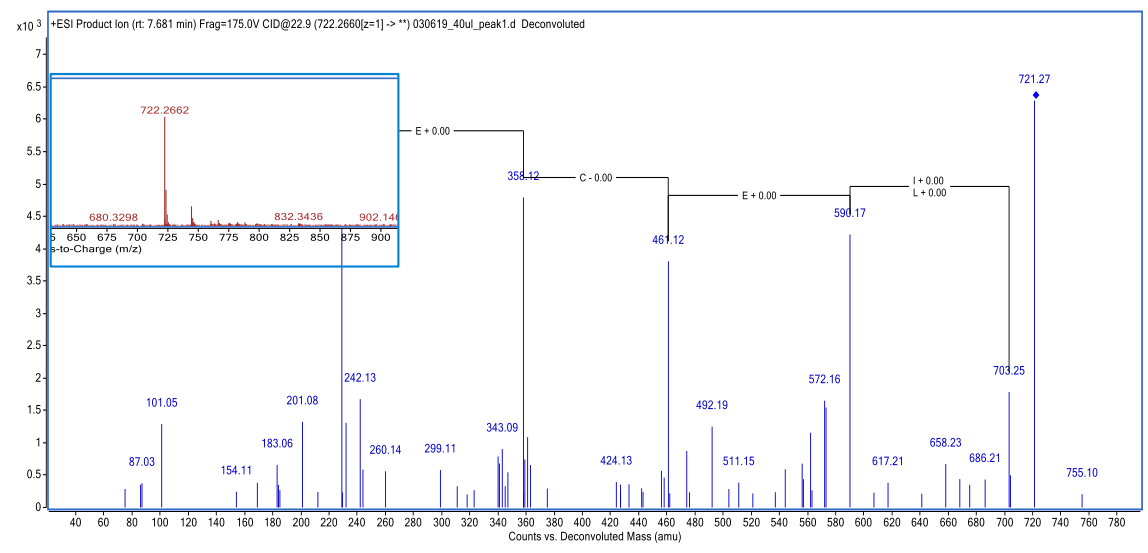

Fig 4c : Deconvoluted MS and MS/MS spectrum of NDECEL (721.2589 Da)

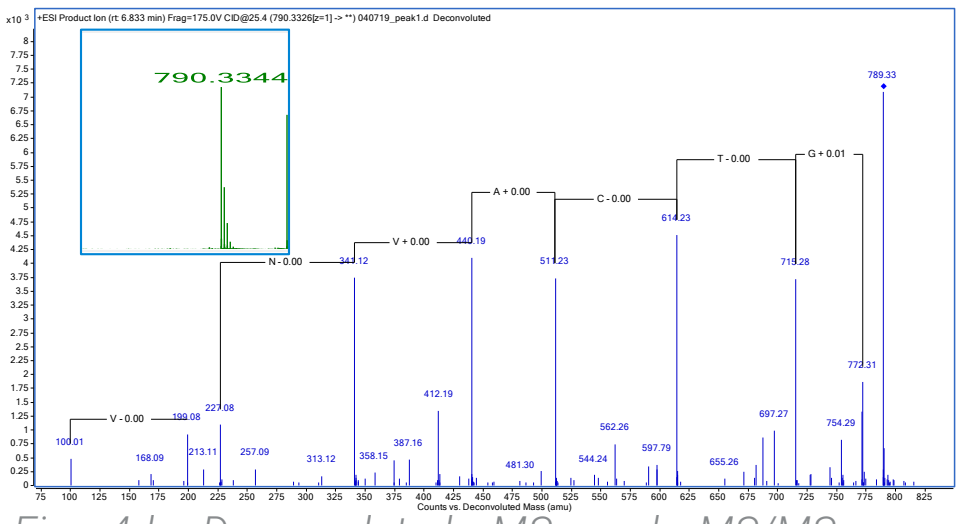

Fig 4d: Deconvoluted MS and MS/MS spectrum of CVNVACTG (789.33 Da)

Confirmation of Cys7-Cys15 linkage

The cleavage of Cys4-Cys12 bond results in two fragments viz. CTGCL (520.1774 Da) and CELCVNVA(874.3677 Da)

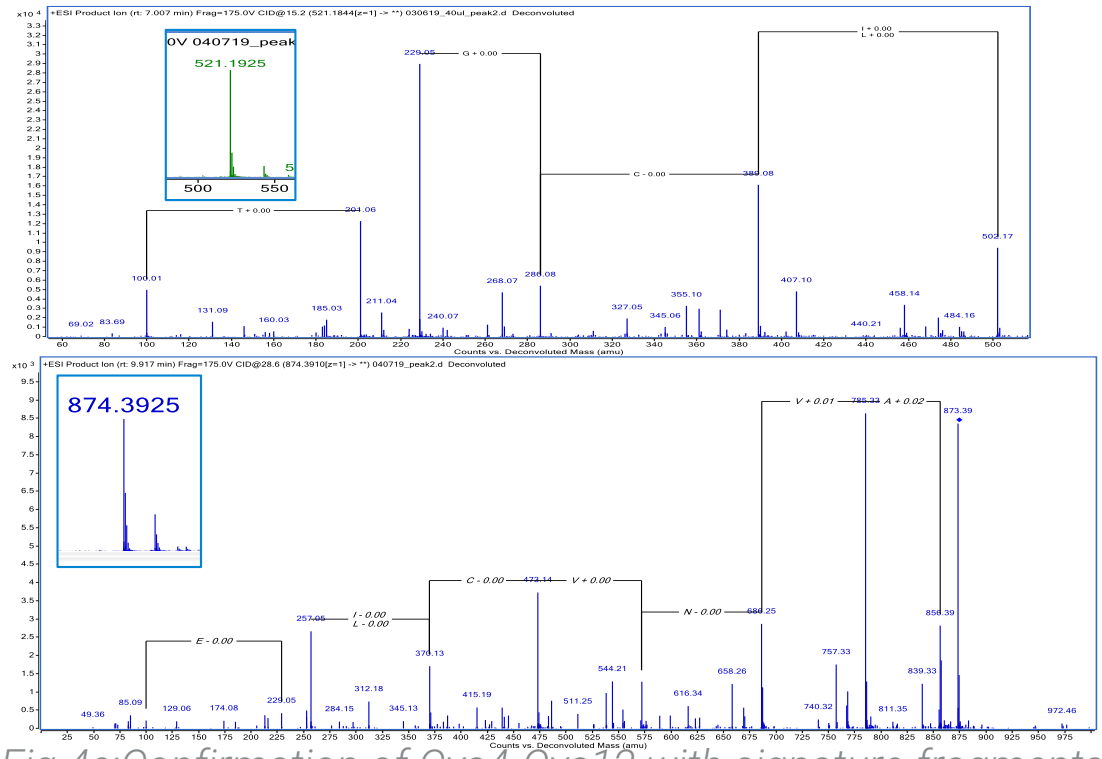

Fig 4e:Confirmation of Cys4-Cys12 with signature fragments

Conclusions

- Presence of three di-sulphide bridges was confirmed in the Linaclotide sample (C1≈C6, C2≈C10, C5≈C13) by using signature peptides resultant of differential cleavage.
- Two di-sulphide bridges at C7-C15 and C4-C12 were confirmed in Plecanatide samples.

References

1: Góngora-Benítez M, Tulla-Puche J, Paradís-Bas M, Werbitzky O, Giraud M, Albericio F. Optimized Fmoc solid-phase synthesis of the cysteine-rich peptide linaclotide. Biopolymers. 2011;96(1):69-80.doi:10.1002/bip.21480.Epub2010 Aug 21.

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