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Impurity Monitoring of GLP-1 Therapeutic Peptide Retatrutide Using a 2D-LC/TOF System

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Introduction

Retatrutide is a next-generation triple agonist (GLP1/GIP/glucagon) and an emerging candidate among multivalent GLP1–based therapeutics.[1] Its structural complexity and diverse impurity profile challenge conventional one-dimensional LC methods, which often lack sufficient resolving power to separate closely related impurities, sequence variants, and synthetic byproducts.

In this study, we demonstrate an Agilent multiple heart-cutting 2D-LC platform that enables rapid online solvent exchange and enhanced chromatographic selectivity, allowing targeted isolation of impurity peaks from a TFA-based first-dimension separation.[2] When coupled with the Agilent 6230C Accurate Mass TOF LC/MS system, the workflow delivers fast, sensitive, and accurate mass profiling under MS-friendly second-dimension conditions, providing a robust solution for comprehensive impurity characterization of GLP-1 peptides such as Retatrutide.

Experimental

Materials

Retatrutide was purchased from Selleckchem, Houston, TX. Retatrutide was reconstitute in water to a final concentration of 2 mg/mL.

Instrumentation

- Agilent 1290 Infinity III 2D-LC system comprised the following modules:
  - 1290 Infinity III Bio Multisampler with Thermostat (G7137A)
  - 1290 Infinity III Multicolumn Thermostat (G7116B)
  - 1290 Infinity III Diode Array Detector (G7117B) with Max-Light cartridge cell (G7117-60020)
  - 1260 Infinity III Bio Flexible Pump (G7131C)
  - 1290 Infinity III Bio High-speed Pump (G7132A)
  - 1290 Infinity Valve Drive (G1170A) with 2D-LC ASM Valve (G5643B)
  - Two 1290 Infinity III Valve Drives (G1170A) with multiple heart-cutting valves (G5642-64000) equipped with 40 µL Bio loops
- Agilent 6230C Accurate Mass TOF LC/MS System

Experimental

LC/MS Analysis

Sample was analyzed on 2D-LC/TOF using MassHunter Acquisition software (v12.2) (Table 1).

Table 1. 2D-LC/MS method.

1st Dimension LC method		
Column	Agilent Altura Peptide Plus column 2.7µm, 2.1 x 150mm (p/n 227215-903)	
Sampler temp.	4 °C	
Injection volume	2 µL	
Mobile phase	A) H_2O, 0.1% TFA B) Acetonitrile, 0.1% TFA	
Flow rate	0.4 mL/min	
Gradient	Time (min)	B (%)
	0.0	30
	2.0	30
	22.0	47
	22.1	90
	24.0	90
	24.1	30
UV Detection	220 nm	
2nd Dimension LC method		
Column	Agilent Altura Peptide Plus column 2.7µm, 2.1 x 50mm (p/n 227205-903)	
Thermostat	80 °C for both 1st and 2nd D columns	
Mobile phase	A) H_2O, 0.1% formic acid B) Acetonitrile, 0.1% formic acid	
Flow rate	0.4 mL/min	
Gradient	Time (min)	B (%)
	0.00	30
	1.5	30
	1.6	80
	2.0	80
	2.1	2
	3.5	2
Post time	0.5 min	
Agilent 6230C Accurate Mass TOF		
Ion source	AJS	
Gas temp.	325 °C	
Drying gas	8 L/min	
Nebulizer gas	30 psi	
Sheath gas temp.	350 °C	
Sheath gas flow	11 L/min	
Capillary	3500 V	
Nozzle	400 V	
Octopole RF	750 V	
Fragmentor	175 V	
Skimmer	45 V	
Acquisition Mode	MS only, 100 – 3200 m/z	

Results and Discussion

1D RP-LC Chromatography: TFA vs. FA

Trifluoroacetic acid (TFA) provides key chromatographic advantages over weaker acidic modifiers such as formic acid (FA) for reverse-phase LC analysis of GLP-1 peptides and is widely used in regulated biopharma QA/QC environments.

Figure 1 presents a head-to-head UV chromatographic comparison of Retatrutide using TFA and FA under identical RP-LC conditions. TFA delivered better chromatographic performance, with improved resolution between retatrutide and related impurities. Three impurity peaks were baseline-separated from the main Retatrutide peak using TFA, whereas one impurity (P2) co-eluted with the main peak when FA was used. This result highlights the enhanced impurity resolution achieved with TFA for GLP-1 peptide analysis compared to the MS-friendly acidic modifier FA on an Agilent Altura peptide plus column.

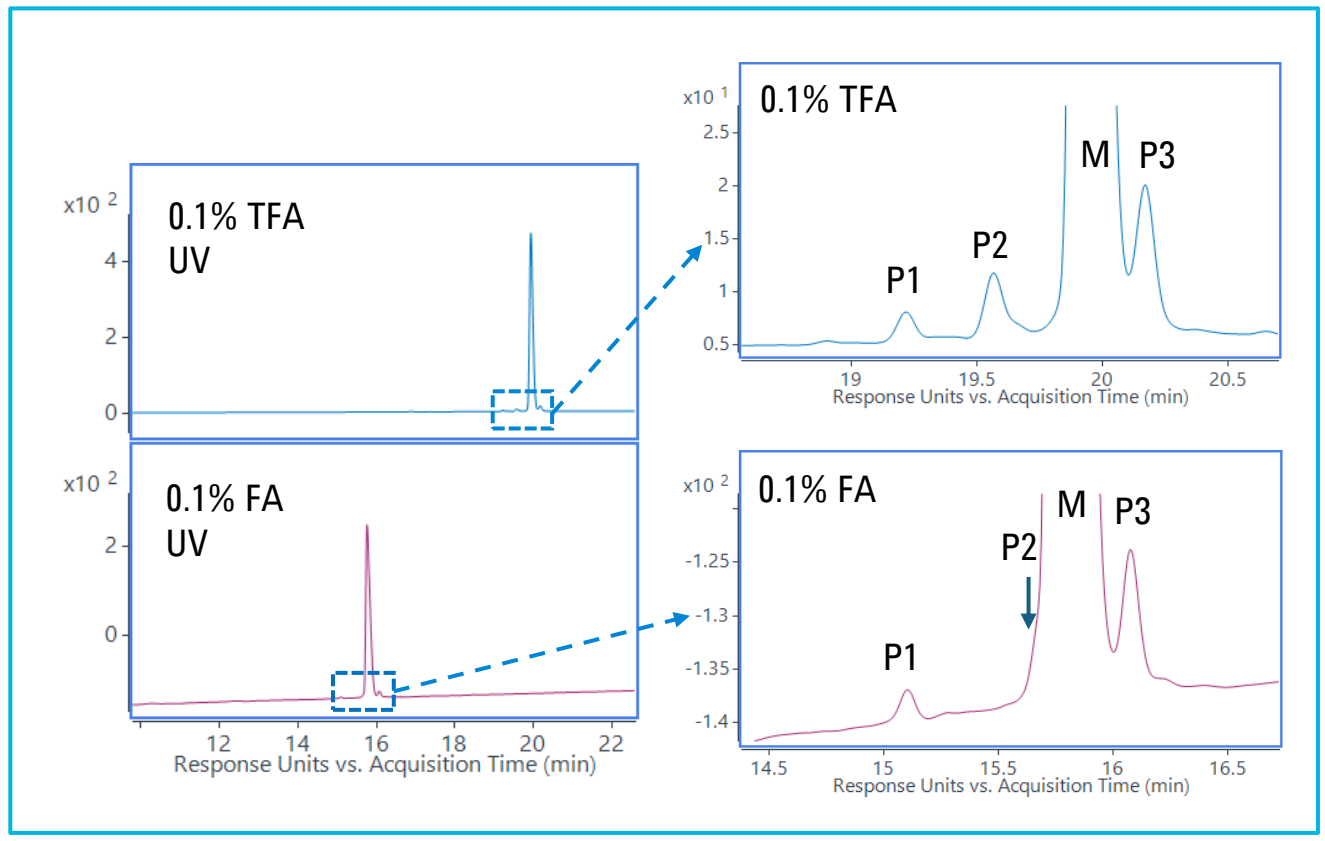

Figure 1. Comparison of 1D RP-LC UV Chromatography using TFA vs. FA as LC solvent modifiers for GLP-1 peptide Retatrutide analysis.

Agilent 2D-LC/TOF System for Retatrutide Analysis

TFA causes strong ion suppression in MS analysis. Agilent 2D-LC coupled to the Agilent 6230C Accurate Mass TOF LC/MS System provides a solution allowing users to use the same TFA-based LC method in the first LC dimension, followed by real time multiple heart-cutting and sample transfer to the 2nd dimension for sensitive and fast LC/MS analysis using FA-based RP-LC method (Figure 2 & 3).

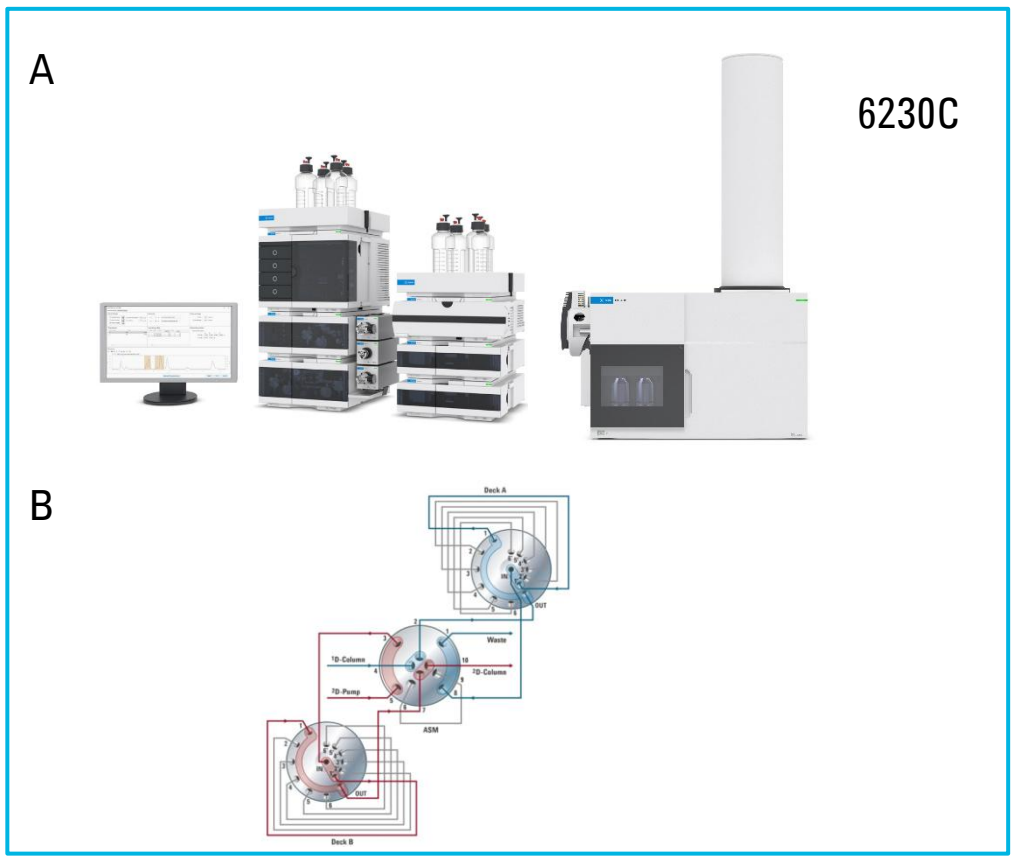

Figure 2. Agilent 2D-LC/MS for Retatrutide analysis. A) Agilent 1290 Infinity III 2D coupled to an Agilent 6230C Accurate Mass TOF LC/MS System. B) Schematic representation of the ASM 2D-LC valve.

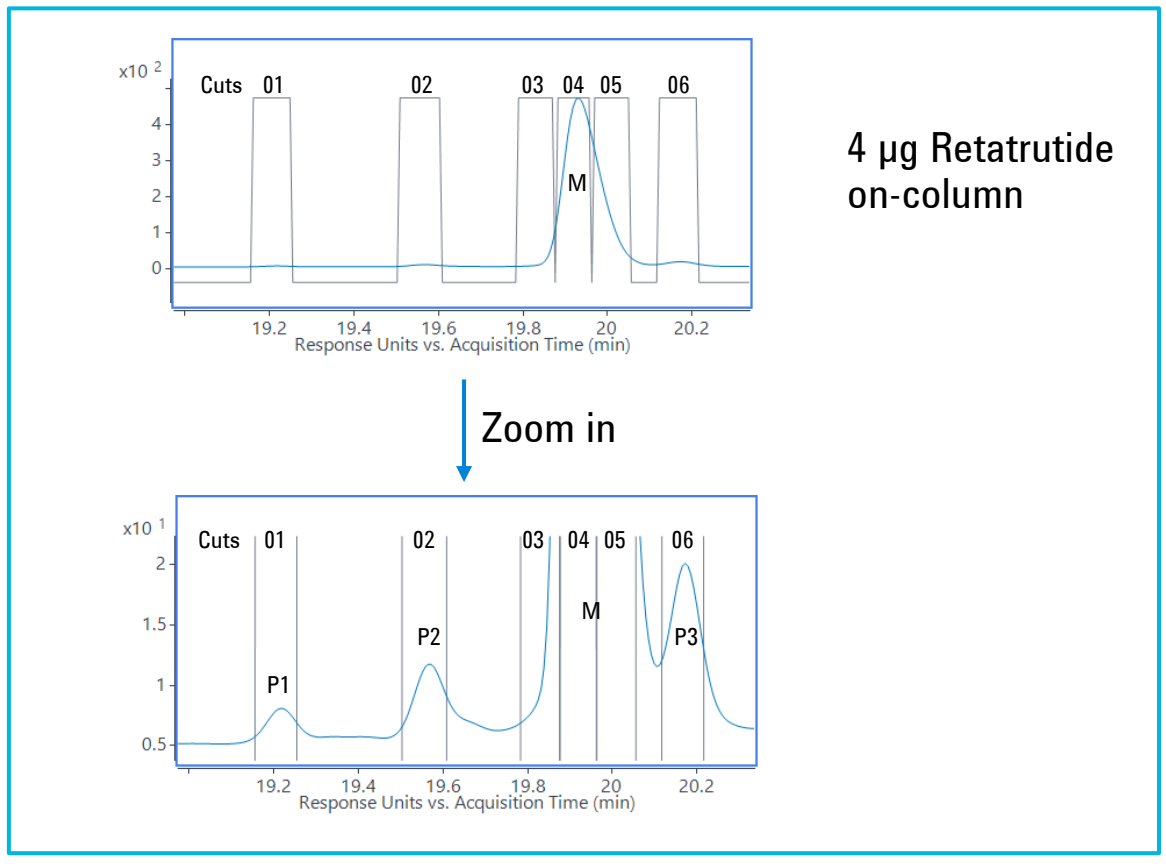

Figure 3. Multiple heart-cuttings of the Retatrutide main peptide peak and the impurity peaks in the first dimension UV-LC chromatogram during 2D-LC analysis.

Results and Discussion

MS Detection of Retatrutide Impurities

The Agilent 6230C Accurate Mass TOF LC/MS System offers sensitive and accurate mass measurement of the low abundant impurities following the 2nd dimension chromatography separation.

Figure 4 shows the representative MS spectra of Retatrutide main peptide (M) and the three impurity peaks (P1-P3) precisely cut from the 1st dimension TFA-based RP-LC separation.

Table 2 summarizes the primary product and three observed impurity peaks, together with proposed explanations for the mass shifts detected in the impurities.

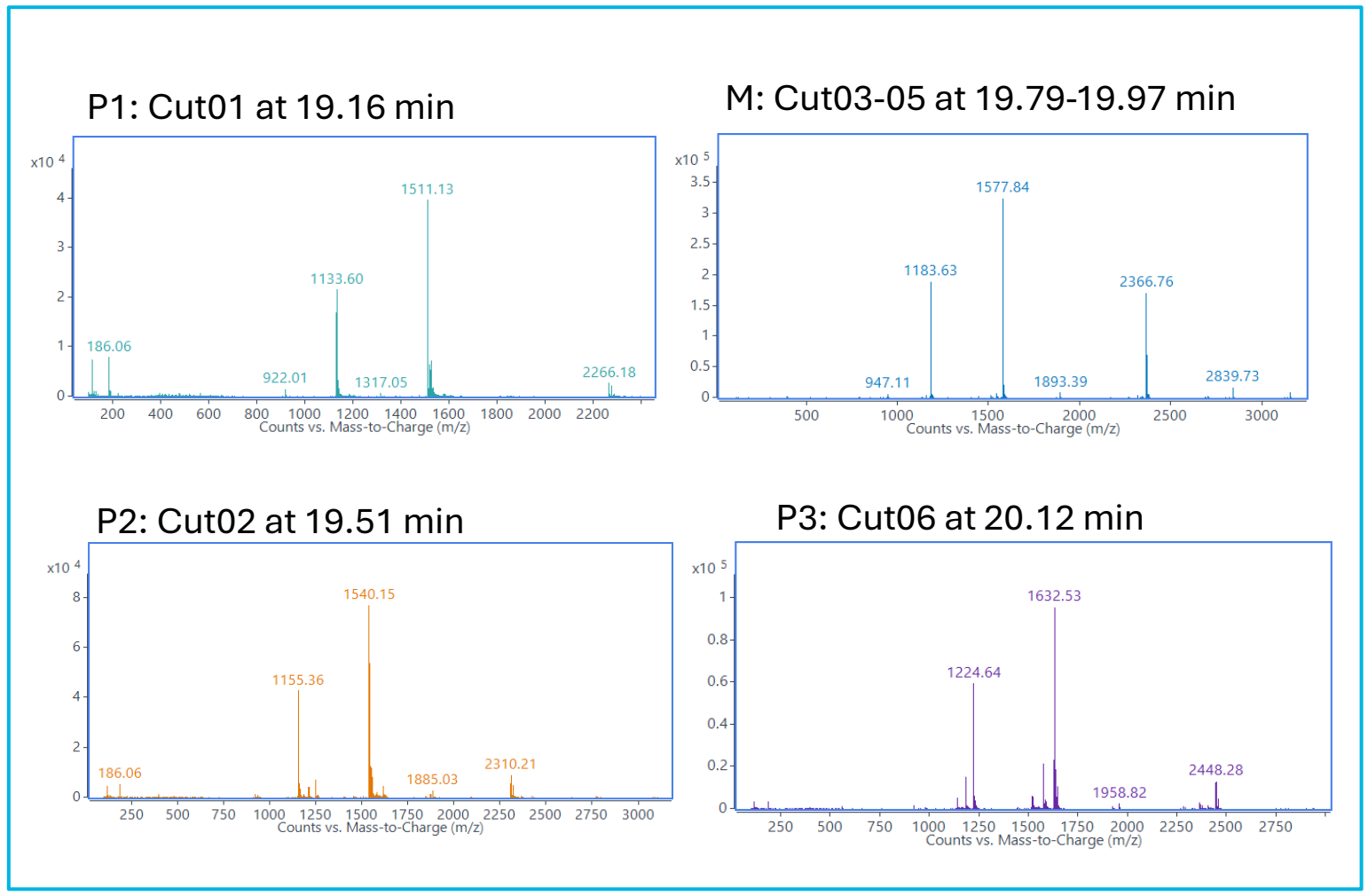

Figure 4. MS spectra of Retatrutide main product (M) and three impurity peaks (P1-P3).

Table 2. Summary of Retatrutide main product and observed impurity peaks.

Peptide Peak	Heart-Cutting Time (min)	%Peak Area (UV)	Deconvoluted Monoisotopic Mass (Da)	Mass Shift from Main Product (Da)	Possible Identity
P1	19.16	0.89	4528.36	-200.13	Deletion of one Serine and one Leucine/Isoleucine
P2	19.51	1.64	4615.42	-113.07	Deletion of Leucine or Isoleucine
M	19.79-19.97	94.58	4728.49	0	GLP-1 peptide Retatrutide main product
P3	20.12	2.90	4891.57	+163.08	Tyrosine insertion

Conclusions

- Agilent 2D-LC with multiple heart-cutting effectively bridges TFA-driven chromatographic performance with FA-compatible MS detection, overcoming ion suppression limitations.
- The integrated 2D-LC/Accurate Mass TOF workflow delivers confident impurity identification and robust characterization, supporting peptide analysis in both QA/QC and development environments.

References

[1] Triple–Hormone-Receptor Agonist Retatrutide for Obesity. Jastreboff, A. M. et al. *N. Engl. J. Med.*, 389, 514–526 (2023).

[2] Confirmation of Peptide-Related Impurity Intact Mass Using 2D-LC. Publication number: 5994-7654EN (2024).

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