Application Note

Biopharma/Pharma

Quantification of Glucagon-Like Peptide-1 Agonist Tirzepatide Using an Agilent 6495D Triple Quadrupole LC/MS System

Suitable for Agilent
1290 Infinity III LC

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Abstract

Synthetic peptide-related impurities generated during manufacturing and storage may affect the safety and efficacy of therapeutic peptides. Glucagon-like peptide-1 (GLP-1) receptor agonists represent the most promising class of synthetic peptide therapeutics. Tirzepatide, a GLP-1 agonist, regulates blood sugar levels and lowers body weight. This application note demonstrates the use of the Agilent 6495D triple quadrupole LC/MS system to measure unmodified and mono-oxidized Tirzepatide. With the multiple reaction monitoring (MRM)-based method, the limit of quantitation (LOQ) for Tirzepatide is 0.025 ng/mL.

Introduction

Peptide-based therapeutics have gained great attention in the research and development of biomedicines. However, peptides can undergo various chemical modifications during formulation, manufacturing, and storage. These changes may impact the efficacy and safety of therapeutic peptides. Therefore, sensitive analytical methods must be used to characterize and quantify these peptide impurities during the biotherapeutic development life cycle. Liquid chromatography-mass spectrometry (LC/MS) is one of the major tools for the characterization and quantification of peptides and their post-translational modifications (PTMs).

Glucagon-like peptide-1 (GLP-1) plays an important role in metabolic regulation and helps in both insulin secretion and the promotion of weight loss.[1] GLP-1 analogs such as Tirzepatide are synthetic versions of GLP-1. Tirzepatide binds to both the glucose-dependent insulinotropic peptide (GIP) and the GLP-1 receptors, acting as a dual agonist. Tirzepatide is an acylated peptide composed of 39 amino acids with an incorporated C_{20} fatty-diacid moiety. Furthermore, this analog achieves a longer half-life compared to native GLP-1. Tirzepatide is approved by the U.S. Food and Drug Administration (FDA) for the treatment of type 2 diabetes mellitus (T2DM).[2]

During the drug development process, monitoring drug stability is crucial to ensure the right dosage for achieving optimal therapeutic levels. Due to greater accuracy and precision, a wide dynamic range, and short method development time, LC/MS has become a popular method to quantitate biomolecules.

This application note demonstrates the quantitative analysis of unmodified/native and oxidized Tirzepatide using an Agilent 1290 Infinity II bio LC system and a 6495D triple quadrupole LC/MS system. Calibration curves were generated to determine the LOQ, and the results showed excellent sensitivity for both peptide forms.

Experimental

Materials and methods

Tirzepatide was purchased from MedChemExpress (Monmouth Junction, NJ). Difluoroacetic acid (DFA), dimethyl sulfoxide (DMSO), and 30% (v:v) hydrogen peroxide (H_2O_2) were procured from Sigma-Aldrich (St. Louis, MO). LC/MS-grade acetonitrile (ACN) and methanol were obtained from Fisher Scientific (Waltham, MA). Ultrapure water was collected from an in-house Millipore Sigma Milli-Q system (Billerica, MA).

Instrumentation

- Agilent 1290 Infinity II bio LC system including:
  - Agilent 1290 Infinity II bio high-speed pump (G7132A)
  - Agilent 1290 Infinity II bio multisampler (G7137A)
  - Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6495D triple quadrupole LC/MS system (G6495D)

Software and data processing

- Agilent MassHunter Workstation acquisition LC/TQ software (12.1)
- Agilent MassHunter Workstation quantitative analysis software (12.1)

Sample preparation

Tirzepatide peptide was dissolved in methanol to a concentration of 1.0 mg/mL. Calibration curve samples were prepared by serial dilution using 30% ACN and 2% DFA. Concentrations of the prepared calibration curve samples ranged from 0.025 to 250 ng/mL. Quality control (QC) samples were prepared at 0.75, 7.5, and 75 ng/mL. For oxidative stress, stock solutions were diluted to 0.5 mg/mL in 30% ACN and incubated with the oxidizing agent H_2O_2 (2% v:v) overnight at room temperature.

LC/MS analysis

Data acquisition was performed using a 1290 Infinity II bio LC system connected to a 6495D triple quadrupole LC/MS system with an Agilent Jet Stream source. The LC separation was performed on an Agilent AdvanceBio Peptide Mapping column (part number 653750-902) using an 8-minute gradient. Tables 1 and 2 list the LC and MS parameters.

Table 1. Liquid chromatography parameters.

Parameter	Value
Instrument	Agilent 1290 Infinity II bio LC system
Column	Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, 2.7 µm, 120 Å
Sample Thermostat	10 °C
Mobile Phase A	0.1% DFA
Mobile Phase B	0.1% DFA in ACN
Gradient	Time (min) %A %B 0.00 70 30 1.00 70 30 4.00 30 70 5.00 10 90 7.50 5 95 8.00 70 30
Stop Time	8 min
Column Temperature	40 °C
Flow Rate	0.4 mL/min

Table 2. MS data acquisition parameters.

Parameter	Value
Instrument	Agilent 6495D LC/TQ system
Ion mode	Positive, Agilent Jet Stream
Gas Temperature	290 °C
Drying Gas Flow	13 L/min
Sheath Gas Temperature	360 °C
Sheath Gas Flow	12 L/min
Nebulizer Gas	35 psi
Capillary Voltage	4,400 V
Nozzle Voltage	0 V
High Pressure Radio Frequency	150 V
Low Pressure Radio Frequency	80 V

Results and discussion

Optimizing the sample preparation, LC, and MS conditions is crucial for enhancing the sensitivity and reproducibility of Tirzepatide quantitation. Different solvents, including methanol, ACN, and DMSO, were evaluated for their ability to dissolve the sample and maintain the stability of the MS signal. Of these solvents, methanol was found to be the most effective diluent. Unmodified and oxidized Tirzepatide forms were well separated with LC using the Agilent AdvanceBio Peptide Mapping column. The MS conditions were optimized using the Method Optimizer feature in the MassHunter Workstation acquisition software.

In this study, Tirzepatide was treated with 2% H_2O_2 to generate the oxidized peptide product.[3] The oxidized peptide represents the potential degradation product that could form in conditions present during storage. Table 3 shows the details for unmodified/native and mono-oxidized Tirzepatide.

Positive electrospray ionization of both unmodified/native and mono-oxidized Tirzepatide produces molecular ion peaks with multiple charge states (3+, 4+, and 5+). The most intense precursor ion signals for unmodified/native and mono-oxidized Tirzepatide are observed at *m/z* 1,204.4 and 1,208.1, respectively, from the $[M + 5H]^{5+}$ ions. Several MRM transitions were observed, and the *m/z* 396.3 y_4 fragment from the 5+ precursor ions was selected as the quantifier (Table 4).

Table 4. Observed MRM transitions for unmodified/native Tirzepatide and mono-oxidized Tirzepatide.

Compound Name	Charge State	Precursor Ion (*m/z*)	MS1 Res	Frag (V)	Product Ion (*m/z*)	MS2 Res	CE (V)
Tirzepatide	5+	963.7	Wide	166	396.3	Unit	22
	4+	1,204.4	Wide	166	396.3	Unit	23
	4+	1,204.4	Wide	166	795.2	Unit	35
	4+	1,204.4	Wide	166	909.5	Unit	35
	3+	1,605.5	Wide	166	396.2	Unit	28
Mono-Oxidized Tirzepatide	5+	966.5	Wide	166	396.2	Unit	23
	4+	1,208.1	Wide	166	396.3	Unit	23
	4+	1,208.1	Wide	166	795.8	Unit	23
	4+	1,208.1	Wide	166	910.1	Unit	23
	3+	1,610.5	Wide	166	369.3	Unit	23

Res = resolution, Frag = fragmentation voltage, CE = collision energy

Table 3. Unmodified/native Tirzepatide and mono-oxidized Tirzepatide.

GLP-1 Peptide	Formula	Molecular Weight (Da)	Sequence
Tirzepatide	$C_{225}H_{348}N_{48}O_{68}$	4,813.55	Y-{Aib}-EGTFTSDYSIXLDKIAQ-{C20 diacid-gamma-Glu-(AEEA)2-Lys}-AFVQ**W**LIAGGPSSGAPPPS
Mono-Oxidized Tirzepatide	$C_{225}H_{348}N_{48}O_{69}$	4,829.55	

Tryptophan (W) oxidation is highlighted in bold

Figure 1 shows the MRM chromatograms for unmodified/native Tirzepatide and mono-oxidized Tirzepatide. These MRMs verify the peptide identity and demonstrate the use of MRM to monitor PTMs such as oxidation. As expected, the oxidized form elutes early (at 3.9 minutes) due to reduced hydrophobicity compared to the native peptide, which elutes later (at 4.0 minutes). The *m/z* 1,208.1 → 396.3 MRM transition shows another peak at 4.3 minutes, corresponding to an unknown signal that was also present in the unmodified/native Tirzepatide sample.

The calibration curve and quality control samples were subjected to quantitative analysis using MassHunter Workstation quantitative analysis software. Figure 2 shows the quantitative performance of the unmodified/native Tirzepatide and the mono-oxidized Tirzepatide. Excellent standard curve fitting is observed for a dynamic range spanning four orders of magnitude (0.025 to 250 ng/mL) for native Tirzepatide and three orders of magnitude (0.25 to 250 ng/mL) for the mono-oxidized Tirzepatide.

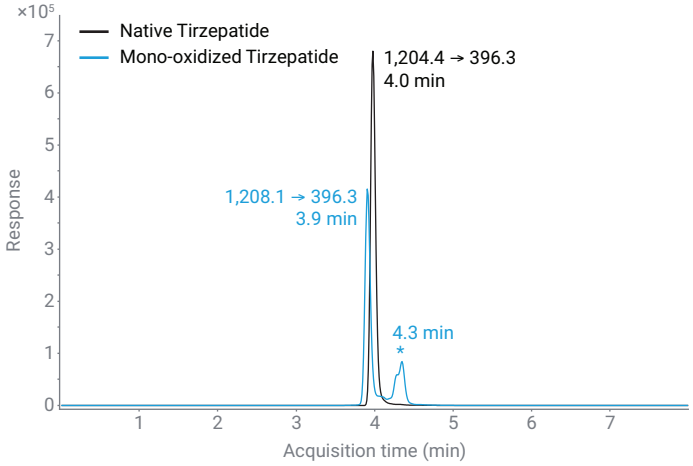

Figure 1. MRM chromatograms of native Tirzepatide (standard, black) and mono-oxidized Tirzepatide (2% H_2O_2-treated, blue). The peak for the native/unmodified peptide transition (*m/z* 1,204.4 → 396.3) occurs at 4.0 minutes, and the peak for the mono-oxidized peptide transition (*m/z* 1,208.1 → 396.3) occurs at 3.9 minutes. An asterisk (*) marks the unknown peak.

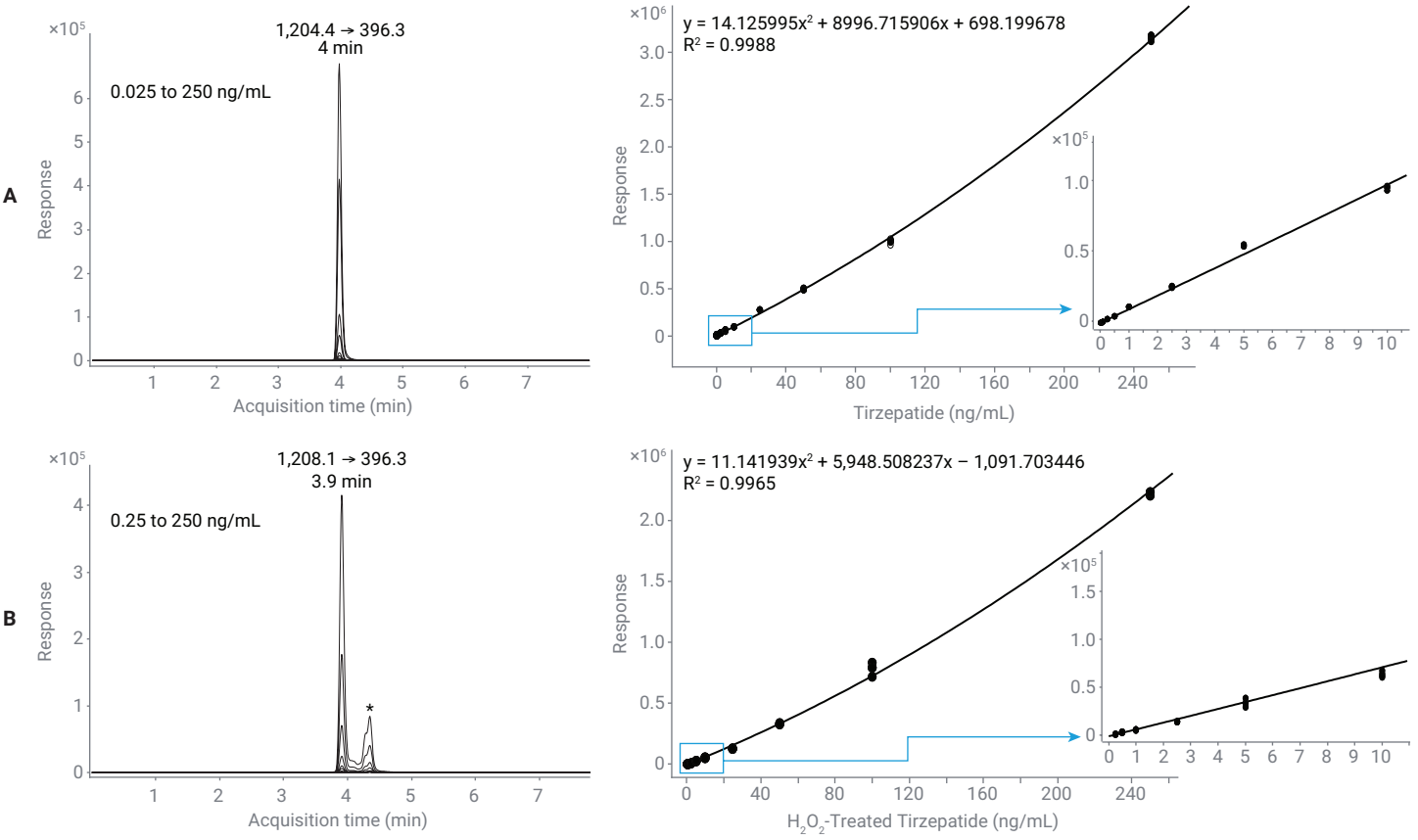

Figure 2. Quantitative performance of (A) unmodified/native and (B) tryptophan mono-oxidized Tirzepatide (n = 4). On the left, overlays of MRM chromatograms for a range of concentrations are shown. An asterisk (*) marks the unknown peak. On the right, the standard curves for each form of Tirzepatide are shown, including insets showing the details of the curve at lower concentration levels.

The correlation coefficients (R^2) were 0.997 and 0.996 for the native and mono-oxidized forms, respectively. Precision and accuracy were excellent at all levels, with percent relative standard deviation (%RSD) < 6% and accuracy ranging from 81 to 115% (Table 5).

Table 5. Precision (area %RSD) and accuracy for the standard curve analysis of unmodified/native (standard) Tirzepatide and tryptophan mono-oxidized (2% H_2O_2-treated) Tirzepatide (n = 4).

Samples	Tirzepatide Standard		2% H_2O_2-Treated Tirzepatide	
Concentration (ng/mL)	Area %RSD	Accuracy (%)	Area %RSD	Accuracy (%)
0.025	2.52	81.37	–	–
0.05	4.28	82.1	–	–
0.1	5.20	81.8	–	–
0.25	4.98	113.7	5.72	117.92
0.5	4.01	101.1	5.97	108.07
1.0	2.06	118.2	5.18	103.05
2.5	3.00	106.3	2.54	90.67
5	1.15	112.7	11.41	97.4
10	1.41	97.6	4.52	89.8
25	0.51	112.9	2.23	82.32
50	1.29	100.2	1.73	99.6
100	1.11	95.6	2.29	107.15
250	0.94	100.4	0.52	99.27

Figure 3 shows MRM chromatograms for blank samples and the lowest concentration levels. This method achieves an LOQ of 0.025 ng/mL for unmodified/native Tirzepatide and 0.25 ng/mL for mono-oxidized Tirzepatide (Figure 3). The method performance was tested with three QC samples at low, middle, and high concentration levels (Table 6). The QC sample precision (percent coefficient of variation, %CV, n = 4) for both native and mono-oxidized Tirzepatide rangeed from 1.01 to 9.55%. At LOQ levels, the %CV for native Tirzepatide (9.55%) and mono-oxidized Tirzepatide (2.43%) were within the acceptance criteria recommended by the U.S. FDA.[4]

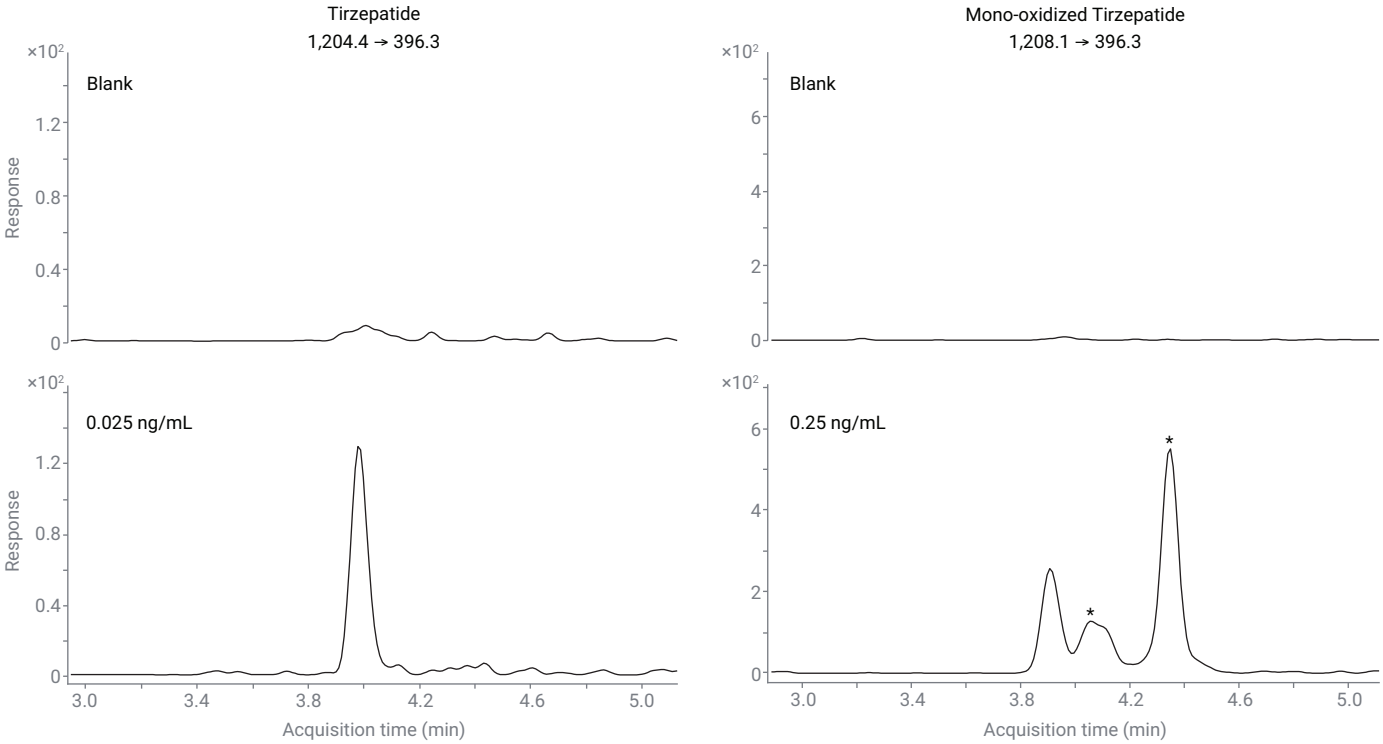

Figure 3. Extracted ion chromatograms of blank samples and samples at the lowest concentration levels. Each unknown peak is marked by an asterisk (*).

Table 6. Precision (%CV) and accuracy results for QC samples (n = 4).

Tirzepatide Standard (ng/mL)	0.025 (LOQ)	0.75 (Low)	7.5 (Mid)	75 (High)
Mean (ng/mL)	0.022	0.82	8.80	82.55
CV (%)	9.55	2.48	1.21	1.01
Accuracy (%)	81.37	100.07	117.3	110.1
2% H_2O_2-Treated Tirzepatide (ng/mL)	**0.25 (LOQ)**	**0.75 (Low)**	**7.5 (Mid)**	**75 (High)**
Mean (ng/mL)	0.29	0.77	8.27	85
CV (%)	2.43	1.82	8.97	3.34
Accuracy (%)	117.92	114.67	109.5	103.05

Conclusion

In this study, a rapid multiple reaction monitoring (MRM)-based method for the highly sensitive quantitative analysis of both native and oxidized Tirzepatide was developed using an Agilent 6495D triple quadrupole LC/MS system coupled with an Agilent 1290 Infinity II bio LC system. The calibration curve demonstrated a wide dynamic range, spanning four orders of magnitude for native Tirzepatide and three orders of magnitude for mono-oxidized Tirzepatide. These results show that this method provides reliable quantitation accuracy and precision.

References

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DE-002783

Printed in the USA, December 16, 2024
5994-7992EN

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