Application Note

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Sensitive Quantitation of Glucagon-Like Peptide-1 (GLP-1) Analog Tirzepatide in Plasma

Using an automated workflow with the Agilent 6495D triple quadrupole LC/MS

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Abstract

Sensitive bioanalytical assays to determine pharmacokinetics and toxicokinetics are crucial for advancing glucagon-like peptide-1 (GLP-1) receptor agonist medications. This application note presents an automated workflow using LC/MS to quantify the GLP-1 analog tirzepatide in human plasma. Our results indicate that this workflow achieved an LLOQ of 0.05 ng/mL for tirzepatide by using 100 µL biological sample, and was linear up to 1,000 ng/mL. This workflow can be used for quantitative analysis of GLP-1 therapeutics without the need for specific antibodies. It also provides excellent sensitivity, high specificity, and fast method development, which all play a crucial role in drug discovery and development.

Introduction

In recent years, the research and application of GLP-1 receptor agonists have significantly increased due to their effectiveness in treating obesity and type 2 diabetes. The global market for GLP-1 analog is projected to grow rapidly, reaching $140 billion by change to 2030. Developing sensitive bioanalytical assays to determine pharmacokinetics and toxicokinetics is crucial for advancing these medications. Liquid chromatography/mass spectrometry (LC/MS) has emerged as an alternative method for analyzing these large molecules due to its high specificity, sensitivity, wide dynamic range, and rapid method development. Additionally, LC/MS can avoid cross-reactivity, enhance productivity, and reduce costs and delays associated with reagent and antigen availability.

Among the notable advancements is the peptide tirzepatide, which is sold under the brand names "Zepbound" for weight loss and "Mounjaro" for diabetes.[1,2] Traditionally, the plasma concentration of tirzepatide is measured by a ligand-binding assay, which requires time to develop antibodies and can lack selectivity and specificity.

In this application note, we present an automated workflow with LC/MS technologies to quantify GLP-1 analog drugs in human plasma using an Agilent 1290 Infinity II bio LC and an Agilent 6495D triple quadrupole LC/MS (LC/TQ) system (Figure 1).

Figure 1. Agilent AssayMAP Bravo protein sample prep platform, 1290 Infinity II bio LC, and 6495D LC/TQ.

Experimental

Materials and methods

Tirzepatide, semaglutide, formic acid (FA), and ammonium formate were purchased from MilliporeSigma (St. Louis, MO, U.S.). Acetonitrile (ACN) and methanol (MeOH) were purchased from Honeywell (Charlotte, NC, U.S.). The 96-well LoBind plates and protein LoBind tubes were purchased from Eppendorf (Hauppauge, NY, U.S.). Also used were Agilent AssayMAP RP-S cartridges.

Instrumentation

The following instrumentation was used:

- Agilent AssayMAP Bravo protein sample prep platform (G5571AA)
- 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 thermostat column compartment (G7116A) equipped with a standard flow Quick Connect bio heat exchanger (G7116-60071)
- Agilent 6495D LC/TQ (G6495DA)

Software

The following software was used:

- Agilent MassHunter Acquisition software (version 12.0)
- Agilent MassHunter Quantitative Analysis software (version 12.0)

Sample preparation

Protein precipitation: A mixture of 150 µL ACN and 150 µL MeOH was added to a 100 µL aliquot of human plasma fortified with different concentrations of tirzepatide. Also, 5 µL of 1,000 ng/mL semaglutide was used as the internal standard. The mixture was vortexed for 2 minutes, then spun down at 17,000 g for 10 minutes. A 350 µL volume of each supernatant was transferred to a 96-well plate and dried down under nitrogen gas with heating. Then, 110 µL water was added to each well to reconstitute. The solution was further purified using AssayMAP RP-S cartridges.

AssayMAP automated purification: AssayMAP RP-S cartridges were conditioned with 80% ACN and equilibrated with water. Then, 100 µL of the previously mentioned reconstituted samples were loaded onto AssayMAP RP-S cartridges at 3 µL/min, and cartridges were washed once with water and once with 10% ACN in 10 mM ammonium formate buffer. The final elution step was carried out by loading 30 µL of 5% FA in 80% ACN to the cartridge at 3 µL/min. Lastly, 30 µL water with 5% FA was added to the final elution and 5 µL was injected into LC/MS for analysis.

LC/MS analysis

Data acquisition was performed using an Agilent 1290 Infinity II bio UHPLC coupled to an Agilent 6495D LC/TQ with the Agilent Jet Stream electrospray ion source. Separation was obtained with an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 120 Å, 2.7 µm). Tables 1 and 2 list the LC and MS parameters used for this workflow. Positive electrospray ionization of tirzepatide yielded $[M + 4H]^{4+}$ signal at *m/z* 1,204.4 as the most intense ion. MRM transitions were optimized and 1,204.4 → 396.2 was chosen as the quantifier and 1,204.4 → 299.2 was chosen as the qualifier, and the semaglutide MRM transition (1,029.4 → 1,238.1) was chosen. Both peptides' MRM transitions are listed in Table 3 with optimal collision energies.

Table 1. LC parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping 120 Å, 2.1 × 150 mm, 2.7 µM
Column Temperature	80 °C
Injection Volume	5 µL
Autosampler Temperature	4 °C
Needle Wash	3 s in wash port (50:50 water:methanol)
Mobile Phase	A) water + 0.1% FA B) ACN + 0.1% FA
Flow Rate	0.4 mL/min
Gradient Program	Time %B 0 30 3.0 45 5.0 60 5.1 95 8.0 95 8.2 30 10.0 30
Stop Time	10.0 min

Table 2. MS acquisition parameters.

Parameter	Value
Ion Mode	Positive
Gas Temperature	290 °C
Drying Gas Flow	15 L/min
Nebulizer Gas	30 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	6,000 V
Nozzle voltage	2,000 V

Table 3. Tirzepatide and semaglutide MRM transitions.

Peptide	Precursor Ion	Product Ion	Collision Energy
Tirzepatide	1,204.4	396.2	26
Tirzepatide	1,204.4	299.2	65
Semaglutide	1,029.4	1,238.1	30

Data processing

All MS data were processed using Agilent MassHunter Quantitative Analysis software.

Results and discussion

Method optimization for GLP-1 analog peptide quantitative analysis

To improve the sensitivity and reproducibility for GLP-1 peptide quantitative analysis, all the sample preparation steps were evaluated to achieve the best results.

1. Organic solvent composition to precipitate proteins was evaluated, including just ACN, just MeOH, and ACN:MeOH (1:1). The ACN:MeOH (1:1) was found to be the best for tirzepatide and semaglutide extraction (Figure 2).
2. Loading buffer was evaluated, including pure water, and water with 0.1% FA and 10%, 20%, or 40% ACN. Pure water was found to be the best loading buffer for GLP-1 peptides.
3. Washing buffer was evaluated, including pure water, water with 0.1% FA, water with 1% FA, and water with 10 mM NH_4COOH pH 10 buffer, with or without 10% ACN. The combination of water and 10% ACN in 10 mM NH_4COOH pH 10 buffer was found to be the best washing solution.
4. Elution buffers were evaluated, including 10 mM NH_4COOH pH 10 buffer with 30% or 80% ACN, and 80% ACN with 0.1%, 1%, and 5% FA. The 80% ACN with 5% FA produced the best results.

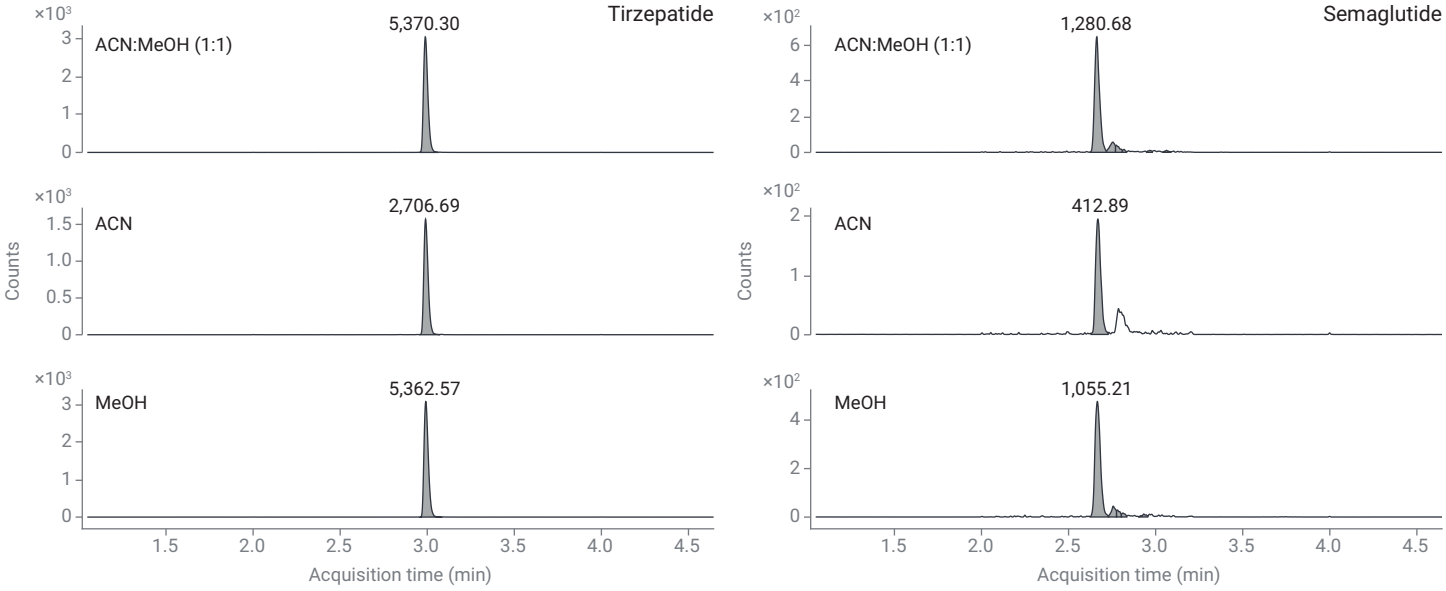

Figure 2. Comparison of organic solvents. Left side is tirzepatide MRM (1,204.4 → 396.2) response after organic solvent precipitation, and right side is semaglutide MRM (1,029.4 → 1,238.1) response after organic solvent precipitation.

LC and MS conditions were all optimized, and the peptides' MRM transitions and collision energies were also optimized to achieve the best MS sensitivity for peptide quantitative analysis. The optimized source parameters are listed in Table 2.

Quantitative analysis of tirzepatide in human plasma

MassHunter Quantitative Analysis software was used to perform quantitative analysis of calibration curve and QC samples. Blank human plasma and tirzepatide had a low limit of quantification (LLOQ) of 0.05 ng/mL, as shown in Figure 3A. The calibration curve was linear up to 1,000 ng/mL with linear fit and $1/x^2$ weight, as shown in Figure 3B. Table 4 shows that all calibration points' precision and accuracy are with ± 15% acceptance criteria, demonstrating excellent assay performance.

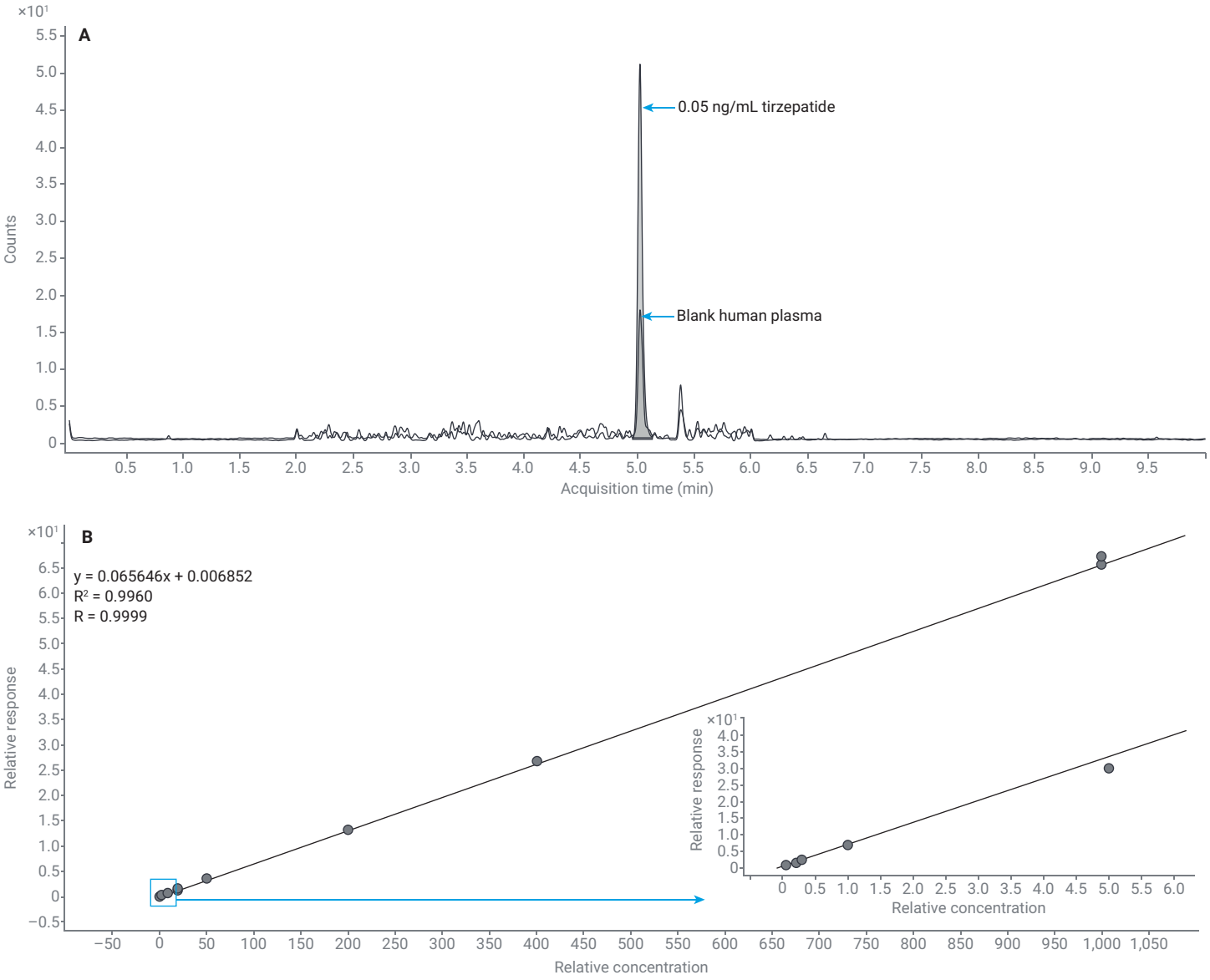

Figure 3. (A) Overlayed tirzepatide MRM (1,204.4 → 396.2) chromatograms of blank human plasma and lowest calibration point. (B) Calibration curve of tirzepatide in human plasma from 0.05 to 1,000 ng/mL. The insert is a zoomed-in snapshot of the lowest five calibration points.

Table 4. Tirzepatide calibration curve (n = 3) results summary.

	Calibration (ng/mL)									
	0.05	0.2	0.3	1	10	20	50	200	400	1,000
Run 1	0.0486	0.183	0.297	1.000	10.324	20.601	51.183	200.032	405.977	1,022.503
Run 2	0.0510	0.179	0.304	0.949	10.326	21.343	50.758	188.363	396.803	941.911
Run 3	0.0512	0.205	0.302	1.109	10.179	19.918	52.995	193.679	385.151	929.260
Mean	0.0503	0.189	0.301	1.019	10.276	20.621	51.645	194.025	395.977	964.558
% Bias	0.51	−5.51	0.36	1.93	2.76	3.10	3.29	−2.99	−1.01	−3.54
% CV	2.92	7.41	1.15	8.02	0.82	3.46	2.30	3.01	2.64	5.24

Table 5. Tirzepatide QC samples' (n = 4) precision and accuracy over three runs.

		QC Concentration (ng/mL)			
		0.05 (LLOQ)	0.1 (Low QC)	5.00 (Mid QC)	800 (High QC)
Run 1	Mean	0.0507	0.0915	5.0159	794.3824
	% Bias	1.3	−8.5	0.3	−0.7
	% CV	9.2	5.3	2.2	1.0
Run 2	Mean	0.0506	0.0997	4.8651	821.4187
	% Bias	1.3	−0.3	−2.7	2.7
	% CV	5.1	5.2	3.2	7.2
Run 3	Mean	0.0518	0.0952	5.350	723.432
	% Bias	3.7	−4.8	7.0	−9.6
	% CV	12.0	8.8	6.5	0.4
Interday	Mean	0.0511	0.0955	5.077	779.744
	% Bias	2.1	−4.5	1.5	−2.5
	% CV	8.5	7.0	5.8	6.8

The intra and interday analytical precision and accuracy of QC samples were determined from three independent runs performed over three days. The precision and accuracy results for tirzepatide in human plasma are shown in Table 4. All levels of QC samples (n = 4) met acceptance criteria of 15%, as recommended by the regulatory agency. The results demonstrate excellent assay performance using automated sample preparation.

Conclusion

The developed GLP-1 peptide quantification workflow, combining automation, the Agilent 1290 Infinity II bio LC, and Agilent 6495D LC/TQ, is an ideal platform for GLP-1 peptide quantitative analysis in biological matrices. The automated LC/MS workflow combines the advantages of both systems and provides excellent assay sensitivity and reproducibility. This workflow achieved an LLOQ of 0.05 ng/mL for tirzepatide by using 100 µL biological sample, and was linear up to 1,000 ng/mL, covering a 4.3 order of magnitude of dynamic range. In three individual qualification runs, the QC samples in all levels met regulatory agency requirements with excellent accuracy and precision. Another advantage of this workflow is that it is universal and can be applied to many other GLP-1 analogs. It also requires minimal method development time to greatly help drug discovery and development.

References

1. Mounjaro- tirzepatide injection, solution. DailyMed. 13 May 2022.
2. Zepbound- tirzepatide injection, solution. DailyMed. 9 Nov. 2023

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