Application Note

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# Sensitive Quantitation of Glucagon-Like Peptide-1 (GLP-1) Analog Semaglutide from Plasma

## Comprehensive workflow for automated sample cleanup and sensitive quantitation


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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 semaglutide in human plasma. Our results indicate that this automated LC/MS workflow can enhance assay sensitivity by five times compared to traditional organic solvent precipitation; this assay achieved LLOQ of 0.2 ng/mL and was linear up to 1,000 ng/mL from 100 µL starting material. 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, glucagon-like peptide-1 (GLP-1) receptor agonists have gained significant attention because of their effectiveness to treat obesity and type 2 diabetes. The global market for GLP-1 analogs is projected to grow rapidly, reaching $140 billion by 2030. Among the notable advancements is the peptide semaglutide, which is sold under the brand name "Wegovy" for weight loss and "Ozempic" or "Rybelsus" for diabetes.[1,2] Developing sensitive bioanalytical assays to determine pharmacokinetics and toxicokinetics is crucial for advancing these medications.

Traditionally, the plasma concentration of semaglutide is measured by a ligand-binding assay, which requires time to develop antibodies and can lack selectivity and specificity. Liquid chromatography/mass spectrometry (LC/MS), which is used for sequence identification of GLP-1 receptor agonists, has emerged as an alternative methodology to precisely quantify these molecules in biological matrices because of its superior sensitivity, wide dynamic range, and high 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 thermostatted 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 an aliquot of 100 µL human plasma fortified with different concentration of semaglutide. Also, 5 µL of 1,000 ng/mL tirzepatide was used as the internal standard. The mixture was vortexed for 2 minutes and 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 then 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 semaglutide yielded $[M + 4H]^{4+}$ signal at *m/z* 1,029.4 as the most intense ion. MRM transitions were optimized and 1,029.4 → 1,238.1 was chosen as the quantifier and 1,029.4 → 136.1 was chosen as the qualifier, and the tirzepatide MRM transition (1,204.4 → 396.2) 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 (p/n 653750-902) |
| 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<br>B) ACN + 0.1% FA |
| Flow Rate | 0.4 mL/min |
| Gradient Program | Time (min) %B<br>0 30<br>3.0 45<br>5.0 60<br>5.1 95<br>8.0 95<br>8.2 30<br>10.0 30 |
| Stop Time | 10.0 min |

**Table 2.** MS acquisition parameters.

| Parameter | Value |
|---|---|
| Ion Mode | Positive |
| Gas Temperature | 270 °C |
| Drying Gas Flow | 13 L/min |
| Nebulizer Gas | 25 psi |
| Sheath Gas Temperature | 200 °C |
| Sheath Gas Flow | 12 L/min |
| Capillary Voltage | 5,500 V |
| Nozzle Voltage | 400 V |

**Table 3.** Semaglutide and tirzepatide MRM transitions.

| Peptide | Precursor Ion | Product Ion | Collision Energy |
|---|---|---|---|
| Tirzepatide | 1,204.4 | 396.2 | 26 |
| Semaglutide | 1,029.4 | 136.1 | 42 |
| 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 semaglutide and tirzepatide 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 5% FA in 80% ACN produced the best results.

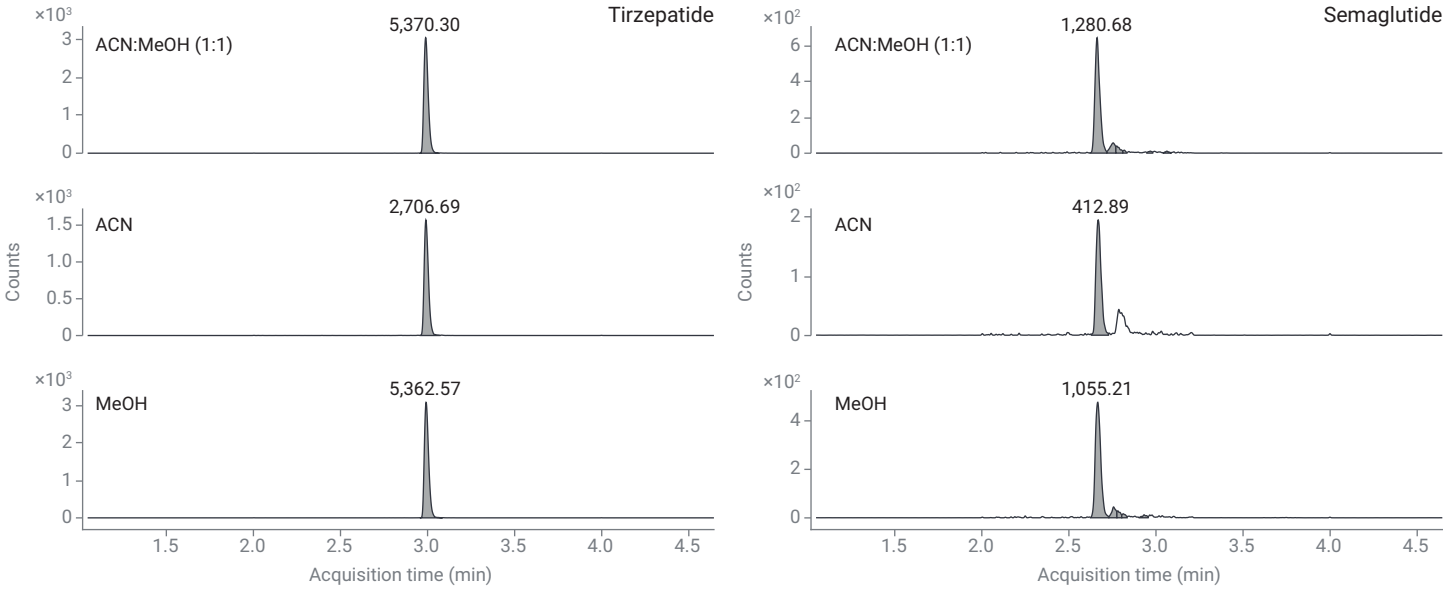


**Figure 2.** Comparison of organic solvents. Left side is tirzepatide MRM (1,204.4 → 396.2) response after organic solvent precipitation, and right side is semaglutide MRM (1,029.4 → 1238.1) response after organic solvent precipitation.

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 semaglutide in human plasma

MassHunter Quantitative Analysis software was used to perform quantitative analysis of calibration curve and QC samples. Overlap chromatograms of blank human plasma and semaglutide low limit of quantification (LLOQ) of 0.2 ng/mL in human plasma are shown in Figure 3A. Figure 3B shows the overlap chromatograms of protein precipitation alone and further purification using AssayMAP RP-S cartridges, which shows that the AssayMAP RP-S cartridges increased assay sensitivity by five fold. The calibration curve was linear up to 1,000 ng/mL with a linear fit and $1/x^2$ weight, as shown in Figure 3C. Table 3 shows that all calibration points' precision and accuracy are with ± 15% acceptance criteria, demonstrating excellent assay performance.

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 semaglutide 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 demonstrated excellent assay performance using automated sample preparation.

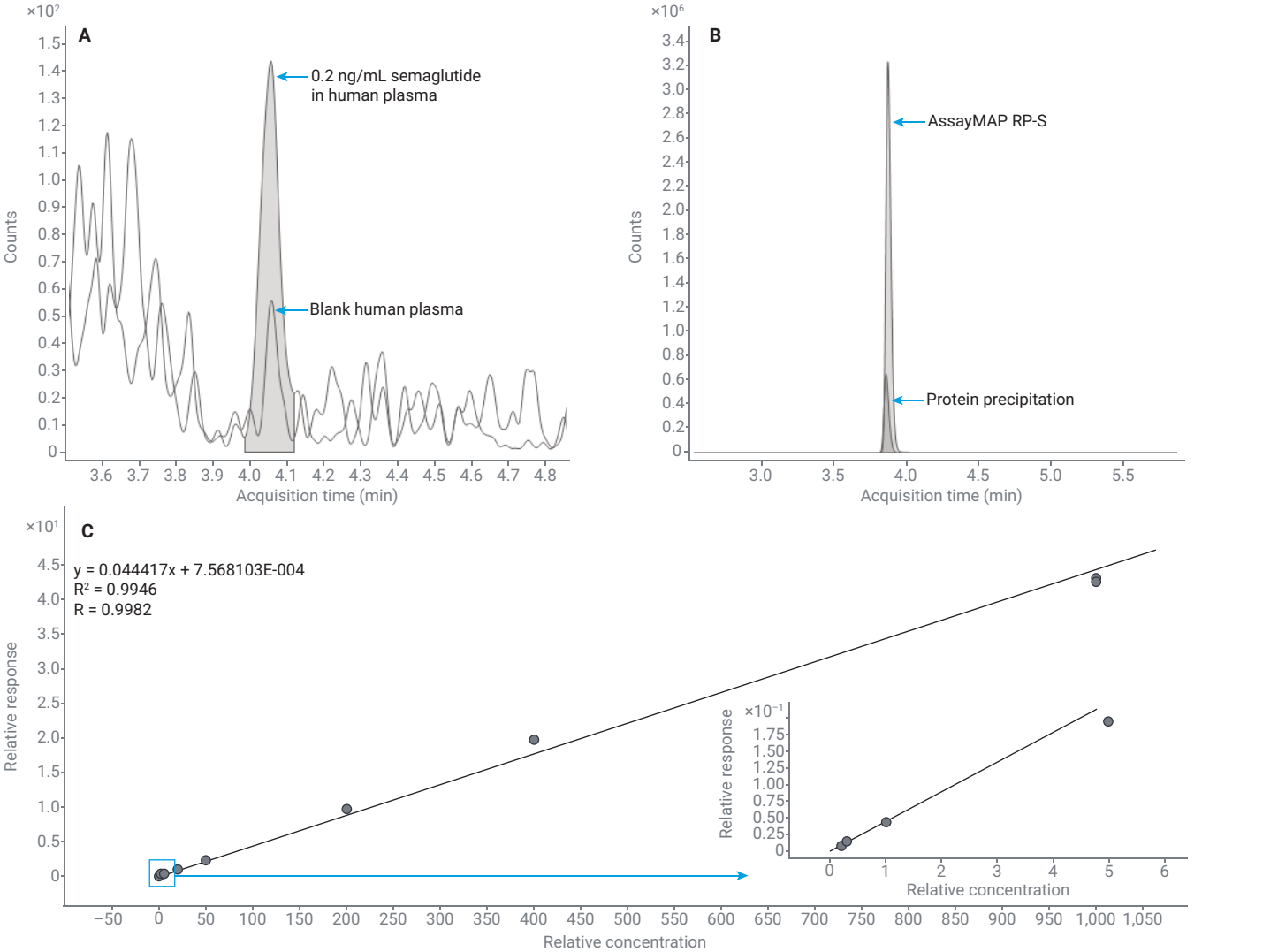


**Figure 3.** (A) Overlap semaglutide MRM (1,029.4 → 1,238.1) chromatograms of blank human plasma and lowest calibration point. (B) Overlap semaglutide MRM (1,029.4 → 1,238.1) chromatograms of protein precipitation and Agilent AssayMAP RP-S cartridge purification for the same concentration of semaglutide. (C) Calibration curve of semaglutide in human plasma from 0.2 to 1,000 ng/mL. The insert is a zoomed-in snapshot of the lowest four calibration points.

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

| | Concentration (ng/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.2 | 0.3 | 1 | 5 | 20 | 50 | 200 | 400 | 1,000 |
| Run 1 | 0.223 | 0.303 | 0.864 | 4.250 | 20.695 | 49.013 | 206.798 | 424.726 | 985.505 |
| Run 2 | 0.186 | 0.279 | 0.975 | 4.385 | 19.75 | 51.588 | 216.095 | 444.826 | 967.528 |
| Run 3 | 0.201 | 0.327 | 0.916 | 4.976 | 18.69 | 50.559 | 213.597 | 434.43 | 995.441 |
| Mean | 0.2034 | 0.303 | 0.918 | 4.537 | 19.712 | 50.387 | 212.163 | 434.661 | 982.825 |
| % Bias | 1.71 | 1.00 | −8.17 | −9.26 | −1.44 | 0.77 | 6.08 | 8.67 | −1.72 |
| % CV | 9.21 | 7.92 | 6.05 | 8.51 | 5.09 | 2.57 | 2.27 | 2.31 | 1.44 |

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

| | | QC Concentration (ng/mL) | | | |
|---|---|---|---|---|---|
| | | 0.2 (LLOQ) | 0.5 (Low QC) | 10.00 (Mid QC) | 800 (High QC) |
| Run 1 | Mean | 0.197 | 0.486 | 8.813 | 809.632 |
| | % Bias | −1.3 | −2.8 | −11.9 | 1.2 |
| | % CV | 6.8 | 4.5 | 1.7 | 2.1 |
| Run 2 | Mean | 0.206 | 0.484 | 10.358 | 821.419 |
| | % Bias | 3.1 | −3.2 | 3.6 | 2.7 |
| | % CV | 2.3 | 5.2 | 3.0 | 7.2 |
| Run 3 | Mean | 0.192 | 0.520 | 9.378 | 871.725 |
| | % Bias | −4.2 | 3.9 | −6.2 | 9.0 |
| | % CV | 7.1 | 7.3 | 9.7 | 3.7 |
| Interday | Mean | 0.198 | 0.497 | 9.516 | 834.259 |
| | % Bias | −0.8 | −0.7 | −4.8 | 4.3 |
| | % CV | 6.7 | 6.3 | 8.8 | 5.5 |

## Conclusion

The developed GLP-1 peptide quantification workflow, combining automation, the Agilent 1290 Infinity II bio LC, and Agilent 6495D LC/TQ system, is an ideal platform for GLP-1 peptide quantitative analysis in biological matrices. Agilent AssayMAP RP-S automated sample cleanup was shown to increase assay sensitivity by five times compared to organic solvent protein precipitation. The developed workflow for semaglutide quantification in human plasma achieved LLOQ of 0.2 ng/mL and was linear up to 1,000 ng/mL from 100 µL starting material, covering a dynamic range of 3.7 order of magnitude. These results are similar or better than reports in the literature. In three individual qualification runs, intra- and interday QC samples' precision and accuracy all met regulatory acceptance criteria, demonstrating excellent assay performance and reproductivity. 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. Ozempic- semaglutide injection, solution. DailyMed. 5 June 2021.
2. Rybelsus- oral semaglutide tablet. DailyMed. 5 June 2021.
3. Wegovy- semaglutide injection, solution. 14 Dec. 2021



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