Poster Reprint

ASMS 2025
Poster number ThP 595

Comprehensive workflow for Automated Sample Cleanup and Sensitive Quantitation of Glucagon-like peptide-1 (GLP-1) Analogs from Plasma

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Introduction

In recent years, research on and the use of glucagon-like peptide-1 receptor (GLP-1R) agonists have surged due to their effectiveness in treating obesity and type 2 diabetes. Among the notable advancements peptide is semaglutide, which is sold under the brand name Wegovy for weight loss, and Ozempic/Rybelsus for diabetes.[1,2,3] Liquid chromatography mass spectrometry (LC-MS) combines the separation of capabilities of liquid chromatography with the detection power of mass spectrometry for precise quantification of these drugs. Here we utilized AssayMAP automated sample preparation to extract semaglutide from human plasma. Our results demonstrate that sample cleanup by AssayMAP RP-S cartridges can improve assay sensitivity 5 times compared to traditional organic solvent precipitation alone.

Experimental

All materials (Acetonitrile, semaglutide, semaglutide, formic acid, 96-well LoBind plates and AssayMAP RP-S cartridge) were purchased from commercial sources,.

A mixture of 150 µL acetonitrile (ACN) and150 µL methanol (MeOH) was added to 100 µL of human plasma aliquot fortified with different concentrations of semaglutide, and a constant amount of tirzepatide as internal standard. The mixtures were vortexed for 2 min and then spun down at 16,000 g for 10 min. 350 µL of each supernatant was transferred to a 96-well plate and dried down under nitrogen gas with heating.110 µL of water was added to each well to reconstitute. The solution was further purified by AssayMAP RP-S cartridges.

RP-S cartridges were conditioned with 80% ACN and equilibrated with water. 100 µL of above reconstitute samples were loaded onto RP-S cartridges at 3 µL/min, cartridges were washed once with water and once with 10% ACN 10 mM ammonium formate buffer. The final elution step was carried out by loading 30 µL of 5% formic acid (FA) in 80% ACN to the cartridge at 3 µL/min. 30 µL of water with 5% FA was added to the final elution and 5 µL was injected into LC/MS for analysis.

All data were collected with 1290 Infinity II Bio LC coupled to 6495D LC/TQ system (Table 1 and Table 2). Semaglutide MRM transition (1029.4→1238.1) was chosen as quantifier with optimal collision energy. Tirzepatide MRM transition (1204.4→396.2) was also optimized.

Experimental

Figure 1. AssayMAP Bravo Platform and 6495D LC/TQ system.

Table 1. UHPLC gradient and conditions

LC Conditions	
Column	AdvanceBio Peptide Mapping 120Å, 2.1 x 150 mm, 2.7 µm
Column temperature	80 °C
Injection volume	5 µL
Autosampler temp	4 °C
Needle wash	5 seconds in wash port (50:50/water: MeOH)
Mobile phase	A = Water + 0.1% FA B = ACN + 0.1% FA
Flow rate	0.5 mL/min
Gradient program	Time %B 0 30 3.0 45 5.0 60 5.1 95 8.0 95 8.2 30 10 30
Stop time	10 min

Table 2. AdvanceBio 6495D TQ MS conditions.

MS Conditions	
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	5500V
Nozzle voltage	400V

Results and Discussion

All sample preparation steps were evaluated to achieve the best sensitivity for semagluatide quantification in human plasma.

- First, organic solvent composition to precipitate proteins was evaluated, ACN : MeOH (1:1) was found out to be the best for semaglutide and tirzepatide extraction.
- Second, loading buffer including water, water with 0.1% FA, 10%, 20% and 40% acetonitrile were evaluated, pure water turned out to be the best loading buffer for GLP-1 peptides.
- Third, washing buffer was evaluated, including water, water with 0.1% FA, water with 1% FA, and 10 mM NH_4COOH pH 10 buffer with/without 10% acetonitrile, the combination of water and 10% acetonitrile in 10 mM NH_4COOH pH 10 buffer turned out to be the best washing solution.
- Last, elution buffer were evaluated, including 10 mM NH_4COOH pH 10 buffer with 30% or 80% acetonitrile, 80% acetonitrile with 0.1%, 1% and 5% FA, 5% FA in 80% ACN produced the best results.

AssayMAP purification improved quantification limit by 5 fold compared to protein crash as shown in Figure 2(A). Blank human plasma and semaglutide low limit of quantification (LLOQ) of 0.2 ng/mL is shown in Figure 2 (B), the calibration curve 0.2-1000 ng/mL with quadratic fit and $1/x^2$ weight is shown in Figure 2 (C), and Table 3.

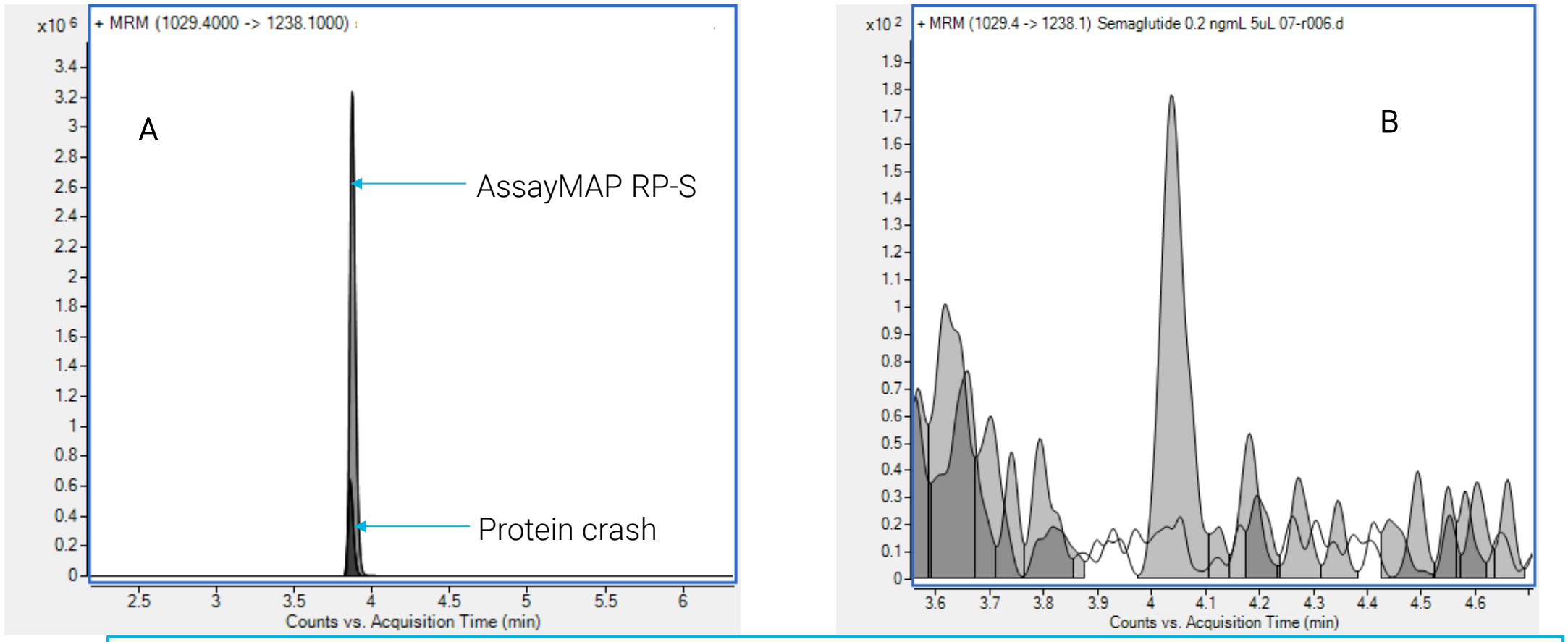

Figure 2. (A) Comparison of semaglutide response after protein crash and AssayMAP RP-S purification. (B) EIC of blank human plasma and lowest calibration point. (C) Calibration curve of semaglutide in human plasma from 0.2 to 1000 ng/mL, insert is zoom in of the lowest four calibration points.

Results and Discussion

The intra-day and inter-day analytical precision and accuracy of quality control samples were determined from three independent runs performed over three days. The precision and accuracy results of semaglutide in human plasma were shown in Table 4. All levels of quality control samples (n=4) met acceptance criteria of 15% as recommended by regulatory agency. The results demonstrated excellent assay performance by using automated sample preparation.

Table 3. Semaglutide calibration curve (n=3) performance over 3 runs.

Calibration (ng/mL)	0.2	0.3	1	5	20	50	200	400	1000
Mean	0.203	0.303	0.918	4.537	19.712	50.387	212.163	434.661	982.825
% Bias	1.7	1.0	-8.2	-9.3	-1.4	0.8	6.1	8.7	-1.7
% CV	9.2	7.9	6.0	8.5	5.1	2.6	2.3	2.3	1.4

Table 4. Semaglutide quality control samples (n=4) precision and accuracy over 3 runs.

	QC conc. (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
Inter-day	Mean	0.1984	0.4966	9.516	834.259
	% Bias	-0.8	-0.7	-4.8	4.3
	% CV	6.7	6.3	8.8	5.5

Conclusions

- An automated LCMS workflow was developed for semaglutide quantification in human plasma. The lower limit of quantification is 0.2 ng/mL and linear up to 1000 ng/mL from 100 µL of 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 qualification runs, intra-day and inter-day QC sample's precision and accuracy all met regulatory acceptance criteria demonstrating excellent assay performance and reproductivity.
- AssayMAP automated sample clean up increase assay sensitivity by 5 times compared to organic solvent protein precipitation.

References

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Published in USA, May 15, 2025

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