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Application Note

SPE-LC/MS Bioanalytical Quantification of the Biotherapeutic Peptide, Semaglutide From Plasma

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Abstract

The following work demonstrates the sensitive, selective, and robust LC-MS bioanalytical quantification of the peptide therapeutic, semaglutide from plasma. Using a combination of selective solid-phase extraction (SPE) sample preparation in the µElution 96-well format, QuanRecovery™ with MaxPeak™ High Performance Surfaces, 96-well plate for sample analysis to mitigate peptide non-specific binding, and LC-MS/MS analysis and quantification using the UPLC™ Peptide CSH™ C_{18} Column, ACQUITY™ UPLC I-Class Plus System, and Xevo™ TQ-XS Mass Spectrometer, to achieve fast, sensitive, and highly reproducible and accurate quantification of semaglutide from plasma biomatrices.

Benefits

- Selective, fast SPE extraction (<30 minutes)
- 96-well µElution plate format for SPE enables concentration of the sample while maintaining solubility and minimizes peptide loss due to adsorption
- Fast UPLC analysis (4-minutes) using the ACQUITY™ Peptide CSH C_{18} Column

- Use of QuanRecovery with MaxPeak HPS sample collections plates, mitigated non-specific binding of Semaglutide, ensuring high peptide recovery, and repeatability during analysis
- Accurate and sensitive quantification of semaglutide with an LLOQ of 0.120 ng/mL from extracted plasma

Introduction

Semaglutide (Ozempic®, Rybelsus®, Wegovy®) is a 32 amino acid, glucagon-like-peptide 1 (GLP-1) analog used in the treatment of type 2 diabetes and as a treatment for obesity.[1] With continued research and development of this peptide and closely related GLP-1 agonist peptides, there is continued need for their robust, sensitive, and selective sample preparation and liquid chromatography mass spectrometry (LC-MS) analysis. However, sensitive and robust quantification of large peptides, like semaglutide (MWT 4114), are often difficult to analyze by LC-MS/MS, as MS sensitivity is low due to poor transfer of the peptide into the gas phase, dilution of signal across charge states, and poor fragmentation. In addition, the semaglutide suffers from significant non-specific binding and poor solubility, making LC and sample preparation method development challenging.

The work described herein takes advantage of a selective µElution reversed-phase solid-phase-extraction (SPE), optimal MS settings and MRM transitions, and a high-efficiency UPLC separation using a column packed with C_{18} particles containing a low-level positive surface charge to provide excellent chromatography peak shape, and use of a low binding sample collection plate to mitigate hydrophobic peptide loss, ultimately facilitating highly sensitive, and robust, low-ng/mL quantification of semaglutide from plasma.

Experimental

Sample Preparation

Semaglutide (Cayman Chemical) was added to commercially available rat plasma. Calibration curve samples concentrations were between 0.120–1000 ng/mL. Semaglutide quality control (QC) samples were prepared at 0.48, 15.6, and 500 ng/mL. Standard curve and QC plasma samples were prepared in triplicate in protein low bind 1.5 mL polypropylene tubes.

Step 1: Protein precipitation (PPT)

200 µL aliquots of the prepared semaglutide rat plasma samples were precipitated with 200 µL methanol and centrifuged for 5five minutes @ 10,000 rcf.

The supernatant was transferred to a 1 mL QuanRecovery 96-well plate containing 400 µL of water and mixed.

Step 2: SPE using an Oasis HLB 96-well µElution PlatePlate

A 3-step (load, wash, and elute) SPE protocol was employed. The entirety (600 µL) of the diluted PPT supernatant samples were loaded onto the extraction plate, followed by a 200 µL wash with 5% methanol. A 2x25 µL elution of the purified sample was performed using a 75/25 acetonitrile/water solution containing 5% formic acid, collected in a QuanRecovery 96-well collection plate. Subsequent analysis of the SPE extracted sample was performed.

LC/MS Conditions

LC system:	ACQUITY UPLC I-Class Plus, Fixed-Loop
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Weak needle wash:	90:10 Water:Acetonitrile with 0.1% formic acid
Strong needle wash:	Acetonitrile:Isopropanol:Water:Methanol (25:25:25:25 v/v/v/v)
Detection:	Xevo TQ-XS
Column(s):	ACQUITY UPLC Peptide CSH C_{18} 100 Å, 1.7 um 2.1 mm x 50 mm (p/n: 186005296)
Column temperature:	65 °C
Sample temperature:	10 °C

Injection volume:	10 µL
Flow rate:	0.3 mL/min

LC Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.300	65.0	35.0	6
0.25	0.300	65.0	35.0	6
3.0	0.300	25.0	75.0	6
3.1	0.300	10.0	90.0	6
3.2	0.300	10.0	90.0	6
3.4	0.300	65.0	35.0	6

MS Settings

Ionization mode:	ESI+
Acquisition range:	MRM
Capillary voltage:	2.00 kV
Cone voltage:	32 V
Desolvation temperature:	500 °C
Desolvation flow:	1100 L/Hr
Cone gas flow:	150 L/Hr
Collision gas flow:	0.15 mL/min

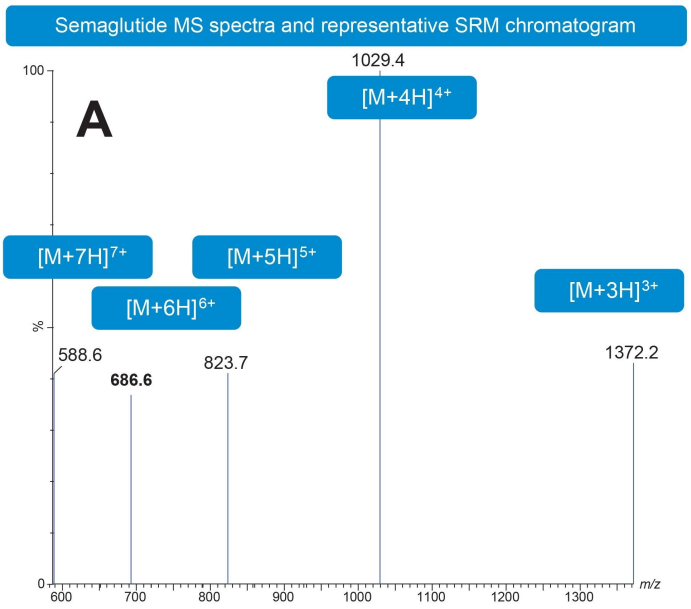
Nebulizer gas flow:	7 Bar

Data Management

Instrument control software:	MassLynx™ (v4.2)
Quantification software:	TargetLynx™

Results and Discussion

LC-MS/MS quantification of semaglutide was performed using an ACQUITY UPLC I-Class coupled to the Xevo TQ-XS MS System operated in ESI+ mode. During MS method development, multiply charged precursors between $[M+3H]^{3+}$ to the $[M+7H]^{7+}$ of semaglutide were observed performing a direct MS infusion of a semaglutide solution (Figure 1A). Following infusion, confirmation of these charge states under LC chromatographic separation and MS detection using Selected Ion Monitoring (SIM) was performed. Under these conditions it was determined that the most sensitive precursors were $[M+4H]^{4+}$ with an *m/z* of 1371.2 and the $[M+3H]^{3+}$ precursor with an *m/z* of 1029 (Figure 1B). During optimization for MRM fragmentation identification, it was found that the $[M+3H]^{3+}$ precursor was harder to fragment, requiring a very high collision energy, and while it had a high MS sensitivity in SIM mode, the fragments evaluated for MRM analysis were > two orders of magnitude less sensitive than the $[M+4H]^{4+}$ precursor and its resulting fragments (Figure 2). For this reason, final MS/MS multiple reaction monitoring (MRM) transitions used for detection and quantification, were 1029.27>1302.6 use for primary quantification, while the 1029.27>1238.1 and 1371.2>1238.1 MRMs were used for confirmation.


Semaglutide MS spectra and representative SRM chromatogram
A
1029.4
[M+4H]4+
[M+7H]7+
[M+5H]5+
[M+6H]6+
[M+3H]3+
588.6
686.6
823.7
1372.2
100
%
0
600
700
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1200
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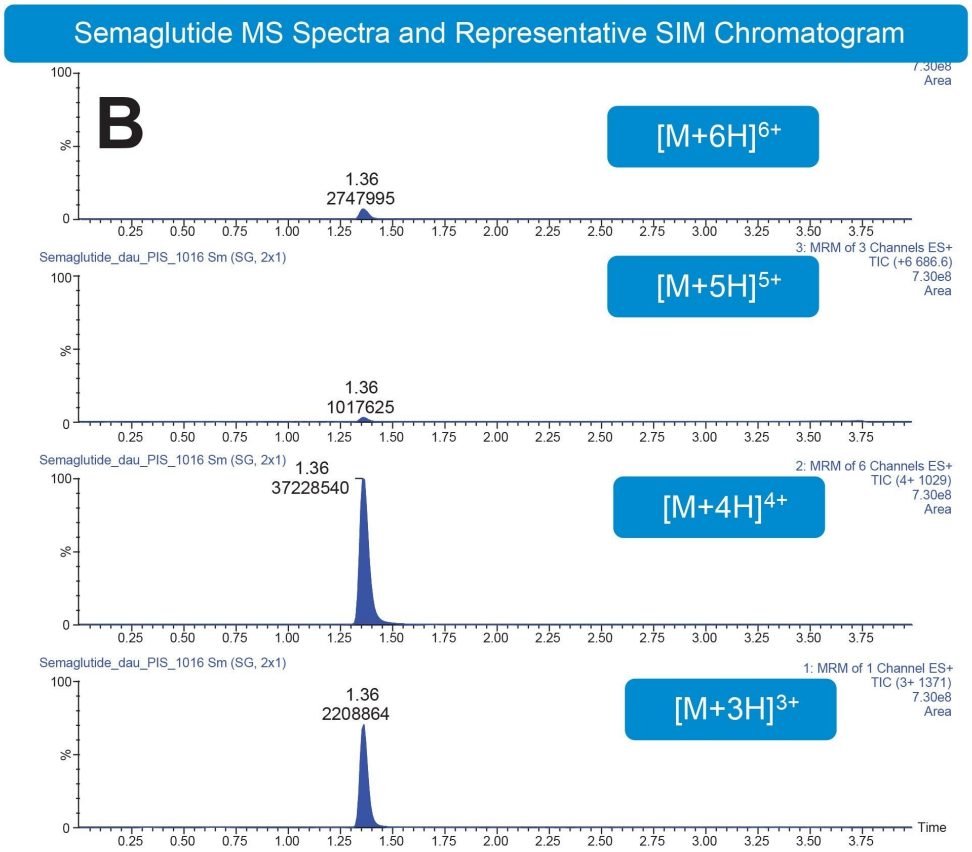

Figure 1. MS spectra (A) highlighting the $[M+3H]^{3+}$ - $[M+7H]^{7+}$ multiply charged precursors of semaglutide and representative single reaction monitoring (SRM) chromatograms highlighting MS response for the $[M+3H]^{3+}$ - $[M+6H]^{6+}$ multiply-charged precursors of semaglutide (B).

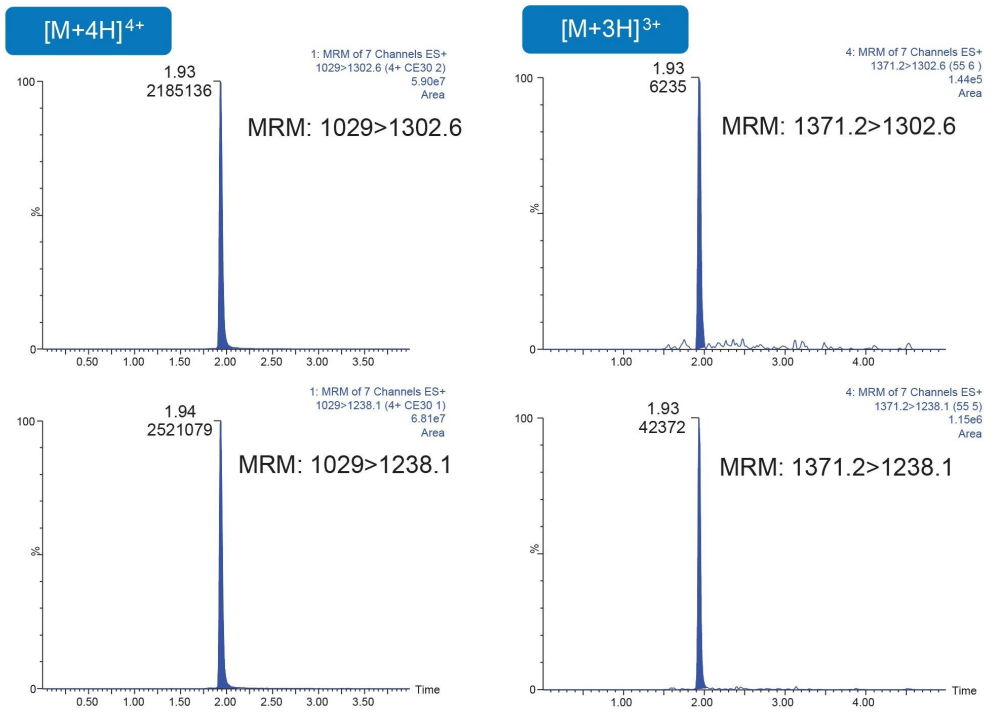

Figure 2. Representative semaglutide MS MRM chromatograms of the $[M+3H]^{3+}$ and $[M+4H]^{4+}$ multiply charged precursors, highlighting best sensitivity using the $[M+4H]^{4+}$ multiply-charged precursor and the 1302.6 and 1238.1 m/z fragments.

Semaglutide is a large and hydrophobic peptide with a molecular weight of 4113.5 g/mol and HPLC Index of 89.7 (relative measure of hydrophobicity). Chromatography of large hydrophobic peptides is often challenged with poor peak shape, peak tailing, and peptide carry-over, with root causes often associated with poor solubility, poor diffusion in and out of the chromatographic pore, and sub-optimal column temperature. To alleviate these issues, column temperatures were maintained at 65 °C, and lower flow rates (between 0.3–0.5 mLs/min) were employed. Using lower flow rates and elevated column temperature improved semaglutide resonance time, facilitating diffusion of semaglutide in and out of the chromatographic pores, while elevated temperatures reduced peak tailing and minimized column carry-over. One of the biggest improvements with chromatography was in the evaluation of column sorbents. This evaluation focused on an ACQUITY UPLC Peptide BEH C_{18} (300 Å) 1.7 µm Column and the CSH ACQUITY UPLC Peptide CSH C_{18} Column (130 Å) 1.7 µm Column, both well-documented for excellent peptide separation performance. The CSH column packed with C_{18} particles containing a low-level positive surface charge provided superior chromatography, with a better MS response and reduced

peak tailing of semaglutide. This performance is illustrated in Figure 3. For these reasons, the CSH C_{18} column was used for semaglutide quantitative analysis.

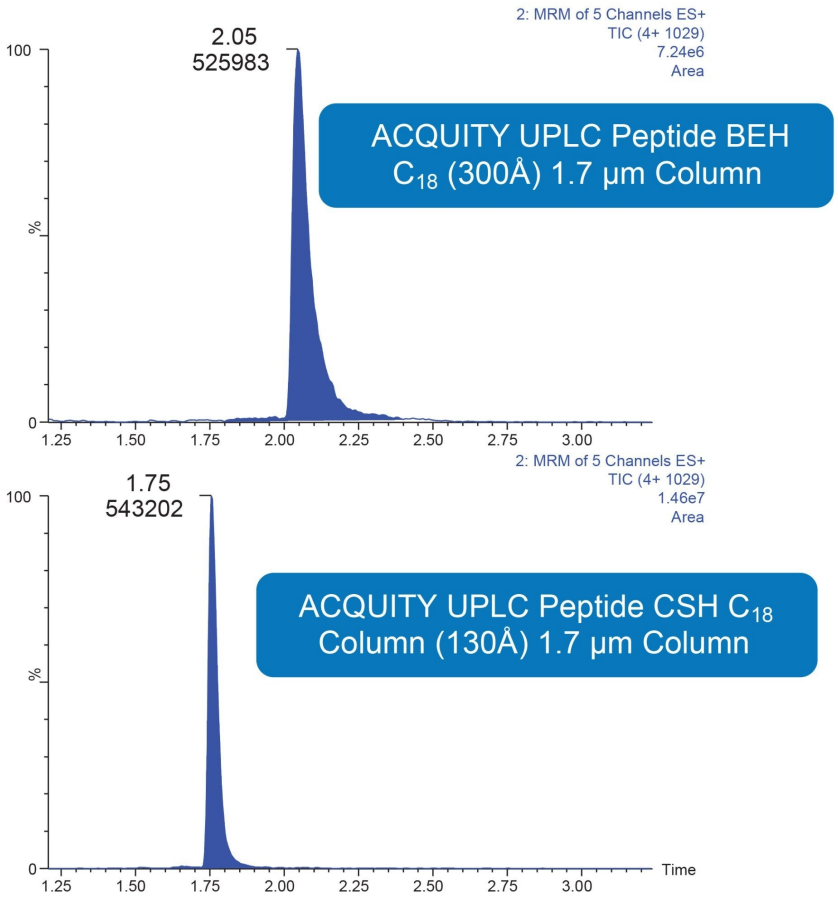

Figure 3. Semaglutide LC column evaluation, comparing the ACQUITY UPLC Peptide BEH C_{18} (300 Å) 1.7 µm Column and the ACQUITY UPLC Peptide CSH C_{18} Column (130 Å) 1.7 µm Column (0.4 mL/min @ 65°C), and demonstrating increased MS response and reduced peak tailing with the Peptide CSH C_{18} Column.

An additional challenge working with semaglutide was encountered during sample preparation and SPE purification due to a high degree of semaglutide non-specific binding (NSB) to consumables and difficulty maintaining peptide solubility throughout this process. As part of the sample preparation and extraction protocol

development, consumables used during sample preparation and LC-MS analysis were evaluated, with key focus on polypropylene consumable designed specifically to mitigate NSB due to peptide hydrophobic interaction. In this evaluation, standard polypropylene and QuanRecovery 96-well sample plates were compared. QuanRecovery plates, with MaxPeak high performance surfaces (HPS) technology were designed specifically to minimize peptide-surface interaction mitigating hydrophobic NSB loss. This comparison, highlighting improved MS response using QuanRecovery vs standard polypropylene plates, using semaglutide solutions (one and ten ng/mL) prepared in a 10% methanol solution, is highlighted in Figure 4. For the same reasons, low-bind polypropylene tubes were also used to further mitigate NSB loss during semaglutide stock solution preparation. Additionally, it was found that bestthe best MS signal was seen for semaglutide stock solutions prepared in a 20–30% methanol solution vs 100% aqueous or 100% methanol solutions.

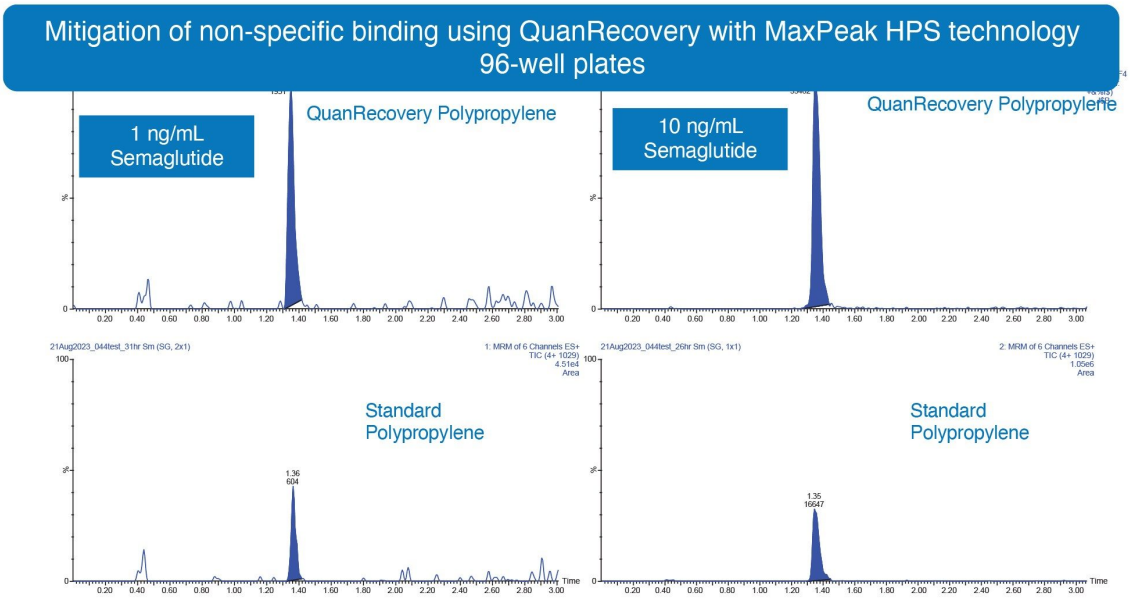

Figure 4. Mitigation of hydrophobic NSB to collection vessels using QuanRecovery™ with MaxPeak HPS 96-well Sample Collection plate, demonstrating improved MS peak response vs standard polypropylene plates.

Sample pretreatment prior to SPE purification proved to be critical in improving recovery and specificity of semaglutide extraction from plasma. Traditional sample pretreatments of acidic and/or basic aqueous solutions, failed to adequately disrupt semaglutide-plasma protein binding, yielding <20% recovery from plasma.

Employing a protein precipitation (1:1) with 5% formic acid methanol pretreatment resulted in 80–100% recovery semaglutide without precipitating the peptide itself. Protein precipitation with higher ratios of organic resulted in peptide loss due to undesired precipitation of semaglutide. Additionally, this, PPT pretreatment further eliminated endogenous interferences from large proteins such as albumin, adding selectivity to this SPE extraction.
Following PPT sample pretreatment, the semaglutide PPT supernatant was diluted 1:2 with water prior to loading onto the Oasis HLB SPE plates. This was done to minimize breakthrough of semaglutide due to high organic composition of the PPT sample. This ensured semaglutide was well retained on the SPE sorbent during the load step, with no breakthrough occurring. The optimum elution solution was determined to be 75% acetonitrile containing 5% formic acid, ensuring optimum elution while maintaining adequate solubility of semaglutide. This final protocol is described in the experimental section and illustrated in Figure 5. Quantitative performance for the calibration curve of semaglutide extracted from plasma is highlighted in Table 1, while QC performance is highlighted in Table 2 and illustrated in Figure 6.

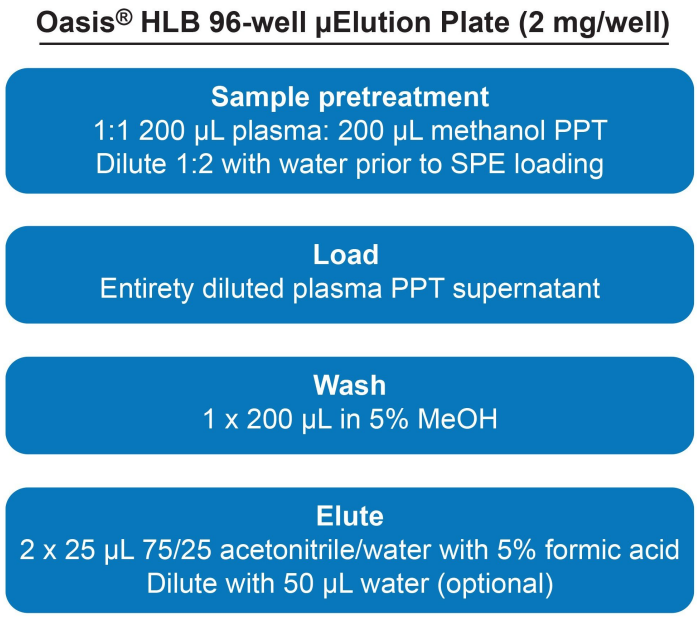

Figure 5. Semaglutide sample extraction protocol using a 1:1 plasma:methanol pretreatment followed by reversed-phase SPE using the Oasis HLB 96-well µElution Plate (2 mg/well), providing 2x sample concentration using a 200 µL starting plasma volume and a 50 µL SPE elution.

Semaglutide rat plasma calibration curve statistics			
Calibration curve dynamic range (ng/mL)	Weighting	Linear fit (R^2)	% Accuracy range
0.12–1,000	1/X2	0.995	-11.6–14.8

Tables 1. Calibration curve statistics of semaglutide extracted from plasma.

Semaglutide rat plasma QC statistics				
QC level	Expected concentration (ng/mL)	Mean observed concentration (ng/mL) (N=3)	Mean % accuracy (N=3)	% RSD
LQC	0.48	0.50	103.80	3.80
MQC	15.6	16.09	103.10	4.28
HQC	500	497.14	99.43	6.04

Tables 2. QC statistics of semaglutide extracted from plasma.

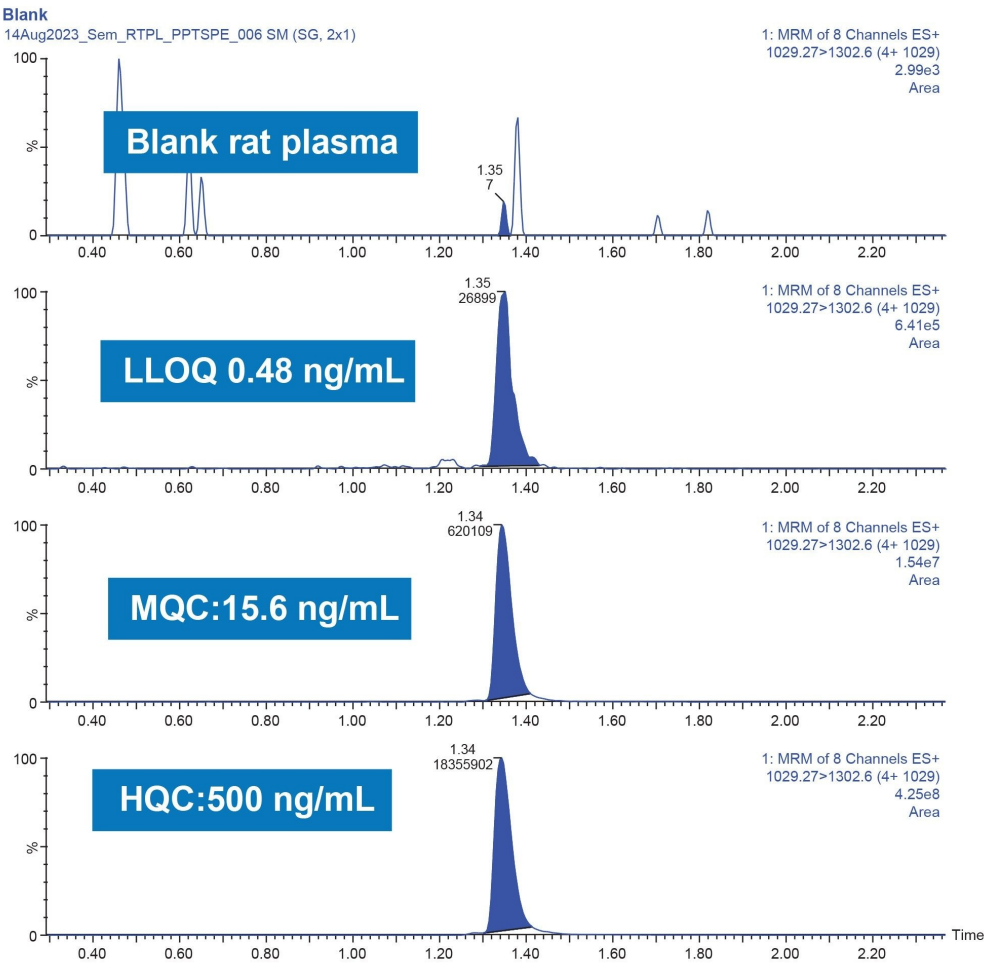

Figure 6. Representative chromatograms of semaglutide QC samples extracted from plasma at 0.48, 15.6, and 500 ng/mL.

Conclusion

The combination of a selective reversed-phase µElution SPE cleanup, mitigation of NSB with QuanRecovery MaxPeak HPS 96-well plates, proper MS fragment choice, and chromatographic separation using the Peptide CSH C_{18} UPLC Column, enabled sensitive, accurate, and robust semaglutide quantification from plasma.

References

1. Semaglutide: Uses, Interactions, Mechanism of Action | DrugBank Online < https://go.drugbank.com/drugs/DB13928> , accessed on 19 September 2023.

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ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

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