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RESEARCH ARTICLE



Development and validation of a sensitive HPLC-MS/MS method for the analysis of semaglutide in human plasma

Christopher Tudan, Joel Stickling, Emily Northwick Darden, Lauren Devine and Shane Needham

Department of Bioanalysis, Veloxity Labs, LLC, Peoria, IL, USA

ABSTRACT

Background: Semaglutide is a human glucagon-like peptide-1 (GLP-1) peptide analog. To date, the literature does not report a validated HPLC-MS/MS assay using a triple-quadrupole mass spectrometer for the quantitation of semaglutide in human plasma in support of pharmacokinetics. A robust, sensitive HPLC-MS/MS assay supporting this class of compounds, is provided and full validation demonstrated.

Methods and results: A triple-quadrupole HPLC-MS/MS method has been developed and fully validated for the quantitation of semaglutide in human plasma, using a stable isotope labeled internal standard and an effective, efficient protein precipitation sample extraction. The method has a sensitive analytical range of 5 ng/mL to 200 ng/mL (and to 1000 ng/mL with a dilution QC) and exhibits excellent accuracy (92.7% to 109.4%) and precision of <10.6%. This highly selective method provided high recovery of the analyte, with minimal matrix effects.

Conclusion: A robust and simple HPLC-MS/MS method to quantify semaglutide in human plasma has been fully validated in accordance with the 2022 FDA M10 guidance. This method is suitable for implementation in clinical studies within the validated analytical range and stability conditions and can be extended to the bioanalysis of other GLP-1 peptide-based therapeutics with similar structural and physiochemical properties.

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GLP-1; semaglutide; peptides; ICH M10; HPLC-MS/MS; method validation

1. Introduction

Therapeutic peptides including glucagon-like peptide-1 (GLP-1) receptor agonists are emerging as a new effective generation of therapeutics because of the advancements in modification chemistry and analytical technologies and rapid progression in proteomics [1–3]. Moreover, peptide drug development has expanded with recent advances in structural biology, recombinant biologics, and new synthetic technologies [3,4]. Peptide-based therapeutics have also attracted attention because of their exceptional specificity, minimal immunogenicity, low toxicity, and therapeutic efficacy [2,5]. Since 2000, more than 30 non-insulin peptide drugs have been approved worldwide [2,3]. Nonetheless, quantitative peptide bioanalysis remains a challenge due to their high molecular weight, complex structures, significant endogenous interference, low *in vivo* concentrations, and susceptibility to nonspecific binding [6]. These aspects of peptides can lead to substantial loss during preparation and increase the risk of matrix interference.

Recently, a new class of GLP-1 receptor agonists has provided excellent therapies for the treatment of diabetes (type 2 diabetes mellitus) and obesity [7–9] and has been demonstrated as potential treatment options for depression [10], major cardiovascular events [7], glaucoma [11], diabetic retinopathy [12,13], and Parkinson's disease [14]. Examples of this class of peptides include semaglutide, dulaglutide, liraglutide, and tirzepatide, to name a few [2,15,16]. Given the

multiple applications of the GLP-1 receptor agonists, there remains a need for continued research on incretin-based therapeutics, such as semaglutide. These GLP-1 peptide analogs were designed to have reduced susceptibility to enzyme degradation and increased *in vivo* half-life while providing effective therapeutic efficacy [17–20]. Semaglutide is a long-acting analog of human GLP-1 with 94% amino acid identity to human GLP-1. The long-acting effect of semaglutide is a result of a covalently linked fatty acid moiety that facilitates binding to albumin resulting in an extended half-life with favorable pharmacokinetic and pharmacodynamic characteristics [21].

GLP-1 receptor agonist peptides exhibit a long half-life with circulatory concentrations in the low nmol/L range [20,22]. Therefore, to accurately describe the pharmacokinetics of these compounds, it is critical to develop a sensitive and robust bioanalytical method to quantify these proteins and their metabolites at very low concentrations in blood. Peptide and protein bioanalytical techniques have traditionally been immunoassay-based assays, such as enzyme-linked immunosorbent assays (ELISA), due to their high sensitivity and high throughput capability [23,24]. However, associated method development for ELISA can be challenging because of the need to identify a specific antibody to the analyte of interest, and moreover, the ELISA has a narrow linear range and potential cross-reactivity when the antibodies cannot structurally discriminate related compounds or analogs [6,15].

Article highlights

- A fully validated bioanalytical method using a triple quadrupole mass spectrometer for the quantitation of the GLP-1 receptor agonist semaglutide is presented.
- The validated method meets the FDA 2022 M10 guidance in method validation and sample analysis.
- A robust and simple sample extraction procedure is described.
- Specific mass spectrometry parameters are outlined.
- GLP-1 receptor agonists such as semaglutide are of significant interest as they are being assessed for many additional therapeutic applications in addition to diabetes and obesity, including depression, major cardiovascular events, glaucoma, and Parkinson's disease to name a few, thus emphasizing the current importance in GLP-1 receptor agonist bioanalysis.

The obstacles associated with ELISA protein quantitation have been overcome with HPLC-MS/MS. Mass spectrometry has, in recent years, become the standard in peptide analysis, taking advantage of the fact that peptides can become hydrolyzed into multiple peptides with smaller molecular weights and then the shorter peptide can be selected based on full scan intensity for quantitation [9,20]. Moreover, today's tandem mass spectrometers are now sufficiently sensitive for peptide quantitation due to their analytical advantages of having a wider dynamic range, greater accuracy, and superior specificity due to a lack of cross-reactivity [15,25]. HPLC-MS/MS has become the primary method for GLP-1 peptide bioanalysis [15–18,25–28], including non-clinical (non-human) assays for semaglutide [5,17,18,29].

There currently exist many ELISA-based assays on the market and used in drug development targeting semaglutide, but there does not exist a published, fully validated HPLC-MS/MS assay for the quantitation of semaglutide in human matrix in the literature. This report presents a novel and robust HPLC-MS/MS assay for quantifying semaglutide in human plasma as well as the associated full validation performed in accordance with the 2022 FDA M10 guidance [30]. This method can be readily adapted for use with other GLP-1 agonist peptide-based therapeutics with similar structural and physiochemical properties.

2. Materials and methods

2.1. Materials

Semaglutide was provided by Cayman Chemical (Ann Arbor, MI, USA), and the internal standard, heavy semaglutide (97% purity) that represents semaglutide containing stable isotope labeling with ^{13}C and ^{15}N so that the mass increase is 40 daltons compared to the parent peptide semaglutide, and having the formula $^{13}\text{C}_{35} \text{ } ^{12}\text{C}_{152} \text{ H}_{291} \text{ } ^{15}\text{N}_5 \text{ } ^{14}\text{N}_{40} \text{ O}_{59}$, was produced and provided by Biosynth (Gardner, MA, USA), ThermoFisher Scientific (Waltham, MA, USA) supplied chemicals including acetonitrile, methanol, dimethyl sulfoxide (DMSO), formic acid, and HPLC grade water. Human plasma (K_2EDTA), lipemic human plasma (K_2EDTA), and human whole blood (K_2EDTA) were supplied by BioIVT (Woodbury, NY, USA). The SiliGuardTM low binding deep well plates (17P687GC) that contain a unique SiliGuardTM treatment that reduces

nonspecific binding were acquired from Analytical-Sales (Flanders, NJ, USA). The Protein Low-Bind tubes (022431102) which are free from surface coating and optimal for potential peptide nonspecific binding were obtained directly from Eppendorf (Mississauga, ON, Canada).

2.2. Preparation of solutions

Individual calibration and quality control (QC) 1.0 mg/mL stock solutions of semaglutide were prepared from powder in 4% formic acid in methanol/water (90:10). A 0.1 mg/mL stock solution of heavy semaglutide internal standard was prepared from powder in DMSO. All stock solutions were stored under refrigeration at 6°C when not in use. The calibration standards were prepared by spiking the 1.0 mg/mL stock solution into human plasma and serially diluting in the Eppendorf Protein LowBind tubes to form an eight-point standard curve consisting of semaglutide concentrations of 5, 10, 20, 40, 120, 180, and 200 ng/mL. QC solutions were prepared similarly by serially diluting the 1.0 mg/mL stock solution into human plasma, with concentrations of 5 ng/mL (lower limit of quantitation quality control, LLOQ-QC), 15 ng/mL (low-quality control, LQC), 60 ng/mL (mid-quality control, MQC), and 150 ng/mL (high-quality control, HQC). The standard and QC solutions were prepared and aliquoted into LoBind polypropylene tubes and stored at -20°C.

2.3. Processing of samples

Control plasma, calibration standards and QCs were thawed at room temperature. Each sample was subsequently vortexed prior to being aliquoted at 50 μL into a 96-well plate. To the sample wells was added 150 μL of 70:30 acetonitrile/methanol containing 50 ng/mL internal standard (IS; heavy semaglutide), except for the matrix blanks (control plasma), to which 70:30 acetonitrile/methanol was added without IS. The plate was sealed and mixed on a plate shaker at 850 rpm for five minutes. The plate was subsequently centrifuged at 3,000 rpm for five minutes at ambient temperature. To each well was added 150 μL of 0.5% formic acid in water/acetonitrile/methanol (50:25:25) to a clean SiliGuardTM low binding deep well plate. Fifty microliters of the supernatant was transferred to the plate and sealed, with subsequent mixing at 850 rpm for five minutes. The plate was then transferred to the HPLC autosampler (set to 6°C) for analysis.

2.4. HPLC-MS/MS conditions

A Sciex QTRAP SCIEX 6500+ mass spectrometer with Analyst (version 1.7.2) software coupled with a Shimadzu Nexera X2 UPLC on a Dell PC workstation was used for HPLC-MS/MS data collection and processing. A sample injection volume of 5.0 μL was injected onto a Phenomenex Kinetex C8, 30 $\times$ 2.1 mm, 100Å, 2.6 μm column, maintained at 40°C. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile; the flow rate of 0.7 mL/min. The acquisition time was 3.6 minutes. The mass spectrometer parameters are provided in Table 1. The

Table 1. Mass spectrometer parameters.

Mass spectrometer	Sciex 6500+						
Ionization/Polarity/Mode	Turbo Ion Spray, positive MRM mode						
Turbo Ion Spray Voltage (IS)	5000						
Turbo Ion Spray Temp (Tem)	500						
Curtain Gas (CUR)	25						
Collision Gas (CAD)	9						
Nebulizing Gas (GS1)	40						
Auxiliary Gas (GS2)	40						
Probe Position	X = 7 mm; Y = 2 mm						
Analyte Name	Parent Ion (m/z)	Daughter Ion (m/z)	Dwell Time (ms)	CE	DP	CXP	EP
Semaglutide	1029.4	1238.2	150	45	80	10	10
Heavy Semaglutide	1039.1	1246.2	150	45	80	10	10

expected retention time for both semaglutide and the IS was 2.3 minutes (± 0.4 mins).

2.5. Assay validation

A detailed method validation was performed in accordance with the current FDA 2022 M10 guidance on method validation and sample analysis [30]. This validation assessed linearity and range, accuracy and precision, selectivity, recovery, hemolysis effect, lipemic effect, matrix effect, carryover, dilution integrity, whole blood (sample collection) stability, freeze/thaw stability, longterm stability, benchtop stability, and reinjection reproducibility. Acceptance criteria are described below.

2.5.1. Calibration curve and range

The calibration curve range is defined by the LLOQ (lower limit of quantitation) and the ULOQ (upper limit of quantitation). Eight concentrations of semaglutide were prepared in human plasma (between 5 ng/mL and 200 ng/mL). One set of eight calibration standards was included in each quantitative batch. Two matrix blanks and two Zero blanks (matrix blank with IS) were analyzed following the highest standards to assess carryover and interference. For the batch to be accepted, at least 75% of the calibration standards were required to have a back-calculated concentration of $100.0\% \pm 15.0\%$ of nominal concentration ($100.0\% \pm 20.0\%$ for the LLOQ). Linearity of the calibration curve was assessed in each quantitative batch. The correlation coefficient (r -value) of the calibration curve needed to be ≥ 0.99 to demonstrate acceptable linearity. A linear regression of $1/x^2$ was applied to all validation batches to produce the best fit.

2.5.2. Accuracy and precision

Intra-batch accuracy and precision were assessed by spiking semaglutide into blank matrix at four concentrations: LLOQ-QC (5 ng/mL), LQC (15 ng/mL), MQC (60 ng/mL) and HQC (150 ng/mL). Each accuracy and precision batch contained six replicates ($n=6$) at each of the QC concentration levels. Freshly prepared calibration standards were used in at least one accuracy and precision batch. The intra-assay accuracy and precision were considered acceptable if the six replicates of QC samples at the LLOQ, low, medium, and high concentrations had a precision (%CV) of $\leq 15.0\%$ ($\leq 20.0\%$ for the LLOQ) and a mean accuracy of $100.0\% \pm 15.0\%$ ($100.0\% \pm 20.0\%$ for the LLOQ). Three individual accuracy and precision batches were

prepared and analyzed to assess inter-assay accuracy and precision of the method.

2.5.3. Selectivity

At least six individual sources/lots of control blank matrix were analyzed without the addition of analyte or internal standard. Any response present in the blank matrix at the corresponding retention time needed to be $\leq 20.0\%$ of the analyte response of the LLOQ and not more than 5.0% of the internal standard response of the LLOQ.

2.5.4. Recovery

The recovery was evaluated by comparing peak areas of extracted samples spiked prior to extraction at the low, medium, and high QC levels ($n=3$) with the peak areas of extracted blank matrix samples spiked post-extraction ($n=3$). The ratio of the mean response of the extracted QC samples to the mean response of recovery samples was expressed as percent recovery. The results of the recovery evaluation were acceptable if the precision (%CV) was $\leq 15.0\%$ for extracted and unextracted replicates at all QC levels and the %CV of the recovery percentages across all concentration levels was $\leq 20.0\%$.

2.5.5. Hemolysis effect

The effect of hemolysis on the method's accuracy and precision was evaluated by analyzing three replicates of LQC and HQCs, each prepared containing 2% hemolyzed blood and analyzed in a quantitative batch. The results of the hemolysis effect evaluation were acceptable if the precision (%CV) was $\leq 15.0\%$ and the mean accuracy was $100.0\% \pm 15.0\%$ of the nominal concentrations of the LQC and HQC.

2.5.6. Lipemic effect

The effect of lipemic plasma on the method's accuracy and precision was evaluated by analyzing three replicates of LQC and HQCs, each prepared in lipemic plasma and analyzed in a quantitative batch. The results of the lipemic effect evaluation were acceptable if the precision (%CV) was $\leq 15.0\%$ and the mean accuracy was $100.0\% \pm 15.0\%$ of the nominal concentrations of the LQC and HQC.

2.5.7. Matrix effect

The matrix effect was evaluated by analyzing three replicates of LQC and HQCs, each prepared using matrix from six different sources/lots. The results of the matrix effect evaluation

were acceptable if the accuracy was $100.0\% \pm 15.0\%$ of the nominal concentration and the precision (%CV) was $\leq 15.0\%$ in all individual sources/lots.

2.5.8. Carryover

Carryover was assessed by injecting matrix blank samples immediately following the ULOQ calibration standard in each batch. Carryover was acceptable if the blanks demonstrate no significant peak area at the retention time for the analytes ($\leq 20.0\%$ LLOQ) and internal standard ($\leq 5.0\%$ LLOQ).

2.5.9. Dilution integrity

Dilution integrity was evaluated by preparing dilution QC samples (DQC) with a semaglutide concentration (1,000 ng/mL) that exceeded the ULOQ and subsequently diluting 10-fold into the analytical range using control plasma. Six replicates were tested in a minimum of one batch. The dilution integrity was acceptable if the precision (%CV) was $\leq 15.0\%$ and the mean accuracy of the dilution QC samples for the dilution factor was $100.0\% \pm 15.0\%$ of the nominal concentration (100 ng/mL).

2.5.10. Whole blood (sample collection) stability

Blank human whole blood (K_2EDTA treated) was pre-incubated for 10 minutes in an incubator set to 37°C . Semaglutide was then spiked into the whole blood at the LQC and HQC concentrations and equilibrated for 10 minutes at 37°C . The spiked whole blood samples were then split into three aliquots. The first whole blood aliquot was placed on wet ice (two-hour wet ice) and the second whole blood aliquot was placed at room temperature (two-hour RT). The third aliquot was processed to plasma immediately (zero-hour) and then placed on wet ice. After two hours, the remaining whole blood aliquots were processed to plasma. All samples were extracted in three replicates per concentration level and analyzed according to the bioanalytical method. The sample collection stability was acceptable if the difference between the mean responses (peak area ratio of analyte to internal standard) of the two-hour (wet ice or RT) and zero-hour samples was $\leq 15.0\%$ at both.

2.5.11. Freeze/Thaw stability

The freeze/thaw stability (F/T) of semaglutide in human plasma was evaluated at -20°C using three aliquots of LQC and HQC samples during five repeated freeze/thaw cycles. Stability QCs were frozen for at least 12 hours between the thawing cycles and thawed for a minimum of one hour at room temperature. Stability QCs for freeze-thaw stability were assessed using freshly prepared calibration standards and QCs for which stability had been proven. The F/T stability was acceptable if the precision (%CV) was $\leq 15.0\%$ and mean accuracy of the stability QC samples at each concentration was $100.0\% \pm 15.0\%$ of the nominal concentration.

2.5.12. Long-term stability

Long-term stability (LTS) of semaglutide in human plasma was evaluated after storing QC samples at -20°C for 97 days. LTS was assessed by assaying three aliquots of LQC and HQC stability samples along with a freshly prepared calibration curve and batch acceptance QCs. The storage stability interval was defined as the difference between the time the QCs were stored and the time the QCs were removed from storage. Note that the

determination of stability at -20°C would cover stability at colder temperatures [30]. The long-term stability was acceptable if the precision (%CV) was $\leq 15.0\%$ and the mean accuracy of the stability QC samples at each concentration was $100.0\% \pm 15.0\%$ of the nominal concentration.

2.5.13. Benchtop stability

The benchtop stability of semaglutide in human plasma was assessed by processing three aliquots of QC samples at LQC and HQC concentrations after storage at room temperature for 24 hours. The benchtop stability was acceptable if the precision (%CV) was $\leq 15.0\%$ and the mean accuracy of the stability QC samples at each concentration was $100.0\% \pm 15.0\%$ of the nominal concentration.

2.5.14. Reinjection reproducibility

Extracted matrix blank, control zero (blank matrix with IS), calibration standards, and six replicates ($n = 6$) of LQC, MQC, and HQC samples from a batch that had met quantitative run acceptance criteria were stored at 6°C (e.g., autosampler or refrigerator) for 94 hours after the end of the original analysis. After this storage period, the batch was reinjected. The reinjection reproducibility was acceptable if the results met the acceptance criteria listed in sections 2.51 and 2.52.

2.5.15. Specificity

The specificity of the method for semaglutide bioanalysis was assessed by performing a mass-based exercise to determine the difference in molecular weight between semaglutide and possible concomitant medications (as described in the drug label information sheet). These medications included Metformin, Warfarin, Digoxin, Atorvastatin, Ethinylestradiol, Levonorgestrel, as well as Acetaminophen, and vitamins B12 and D. None of the possible comedications were within 20 daltons from semaglutide and therefore it was determined that there would be no effect on the analysis of semaglutide in the presence of these comedications.

2.5.16. Stock solution stability

The stability of semaglutide in a 4% formic acid in methanol/water (90:10) stock solution was evaluated at room temperature for at least 6 hours and at 6°C for 7 days. One aliquot of a stock solution was kept at room temperature for 6 hours and another at 6°C for 7 days and then diluted to a concentration for appropriate instrument response. One aliquot of a freshly prepared stock solution was diluted in the same manner. The stability of the stock solutions was considered acceptable if the absolute difference between the mean instrument response (peak area ratio) of the test solution and the freshly prepared solution was $\leq 10.0\%$.

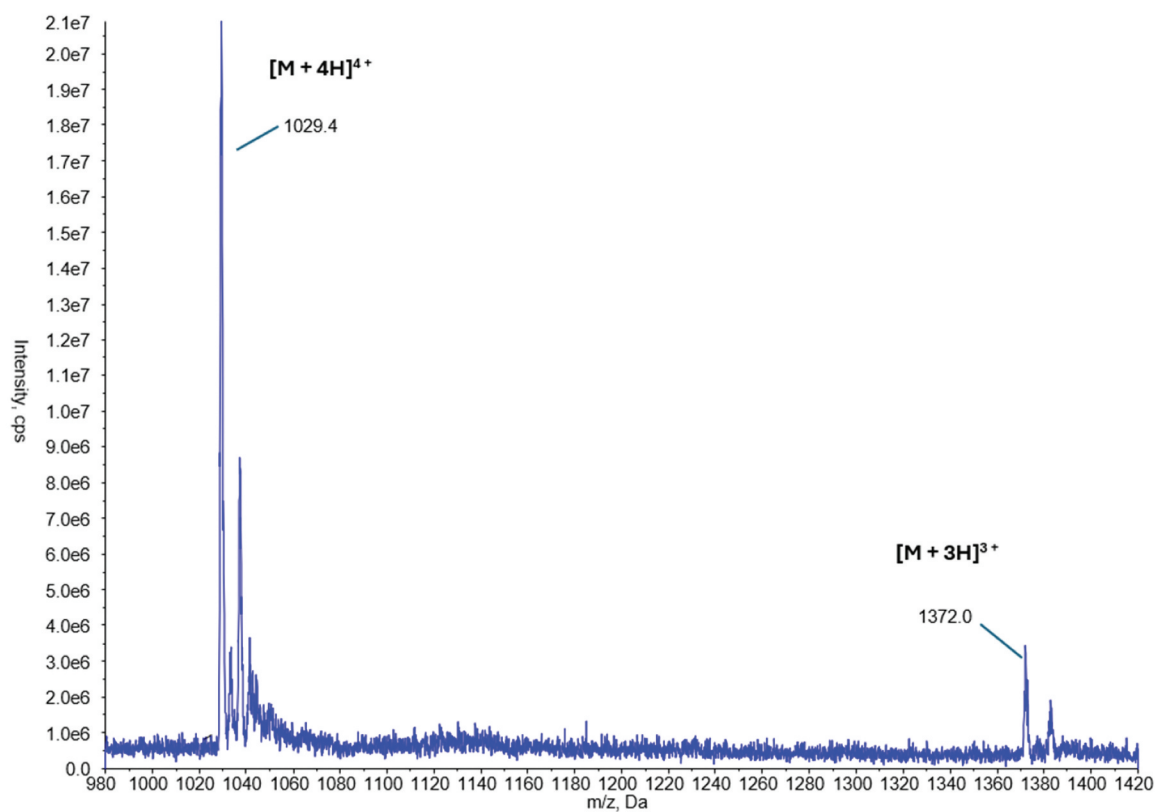
3. Results

3.1. Method validation

3.1.1. Mass spectrometry results

The full Q1 mass scan of semaglutide in the QTRAP 6500+ mass spectrometer is shown in Figure 1(A). As a large molecular weight peptide (m.w. = 4114), multiple charged precursor ions were observed in the full scan of the protonated semaglutide. The most abundant precursor ion in the Q1 mass scan spectrum was

A.



B.

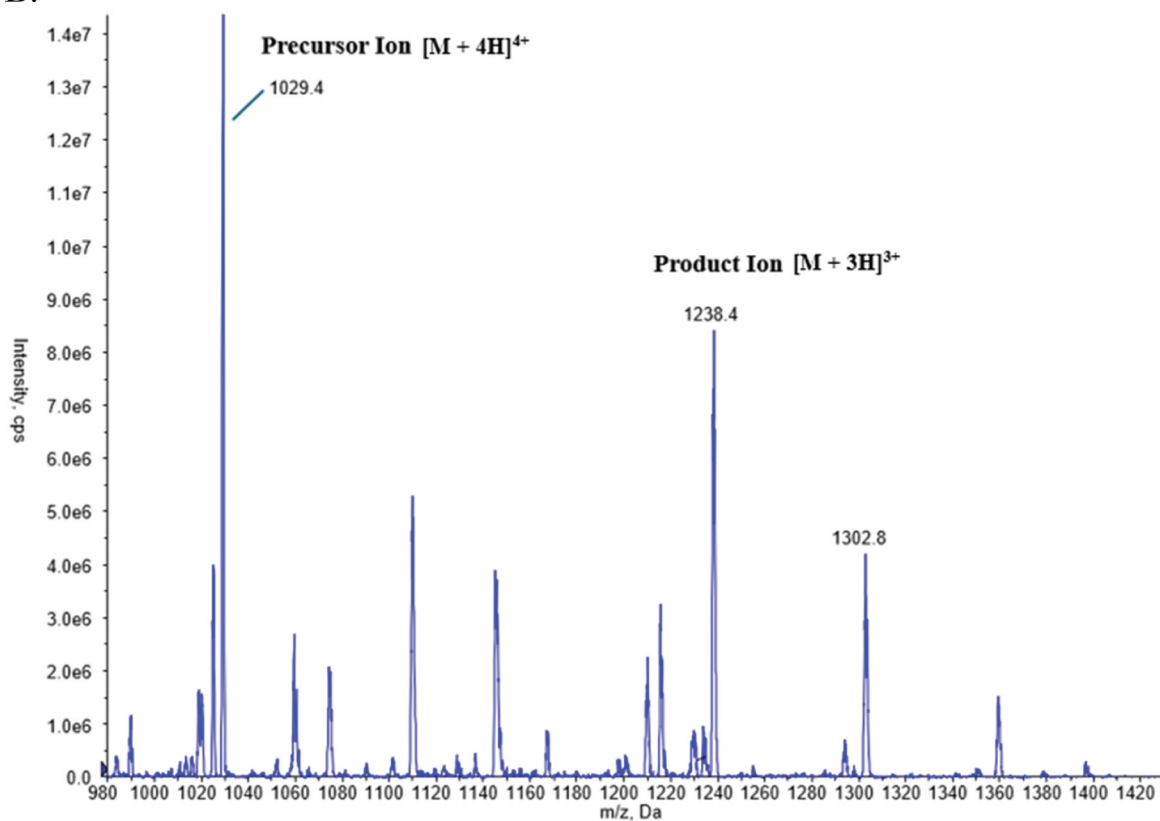


Figure 1. (A) Full Q1 Scan of semaglutide, (B) Product ion spectra of protonated semaglutide.

$[M + 4H]^4+$ at 1029.4 m/z. In the product ion mass spectra of the protonated semaglutide, several product ion fragments were observed (Figure 1(B)). Following analysis, the MRM transitions selected were m/z 1029.4 $\rightarrow$ 1238.2 for semaglutide and m/z 1039.1 $\rightarrow$ 1246.2 for the stable labeled internal standard, as these exhibited the greatest signal intensity with minimum interference for the quantification of semaglutide. The charge state of product ion illustrated in Figure 1(B) was $[M + 3H]^3+$. Figure 2 shows the representative MRM chromatograms of semaglutide and the internal standard. The retention time of both semaglutide and the internal standard was 2.3 minutes.

3.1.2. Calibration curve and range

The calibration curve range was defined by the LLOQ (5 ng/mL) and the ULOQ high standard (200 ng/mL). Eight calibration concentrations within this range were prepared in human plasma and analyzed in singlicate. All standards within the range were well within the defined acceptance criteria with an accuracy and precision range of 97.5% to 103.5% and 1.7% to 4.6%, respectively, as shown in Table 2. The correlation coefficients were all >0.99 , with a range from 0.9984 to 0.9996, reinforcing the reproducibility of the calibration curve.

3.1.3. Accuracy and precision

To measure intra-assay accuracy and precision, semaglutide was spiked into blank human plasma at four concentrations

(LLOQ, LQC, MQC, and HQC). Six replicates of each were subsequently analyzed in three batches. The accuracy across the concentration ranged from 92.7% to 109.4%, and the precision ranged from 2.0% to 10.6% as shown in Table 3. For inter-assay accuracy and precision, three separate analytical runs containing six replicates of QCs at the concentrations listed above were analyzed. The calculated accuracy across the concentrations ranged from 97.3% to 99.6%, as shown in Table 3. Therefore, the analytical method demonstrated acceptable accuracy and precision across the range from the LLOQ-QC to the HQC.

3.1.4. Selectivity

No significant interference from endogenous components in six of the six plasma lots screened was observed at the retention time and mass transition of semaglutide and heavy semaglutide (IS). The percent peak area of the human plasma lots compared to the LLOQ standard ranged from 1.9% to 3.7%.

3.1.5. Recovery

The mean absolute recoveries for semaglutide were calculated as 83.3% for the LQC, 78.7% for the MQC, and 79.7% for the HQC. The recovery for the IS was 75.5%. Recovery was consistent, reproducible, and precise, as shown in Table 4.

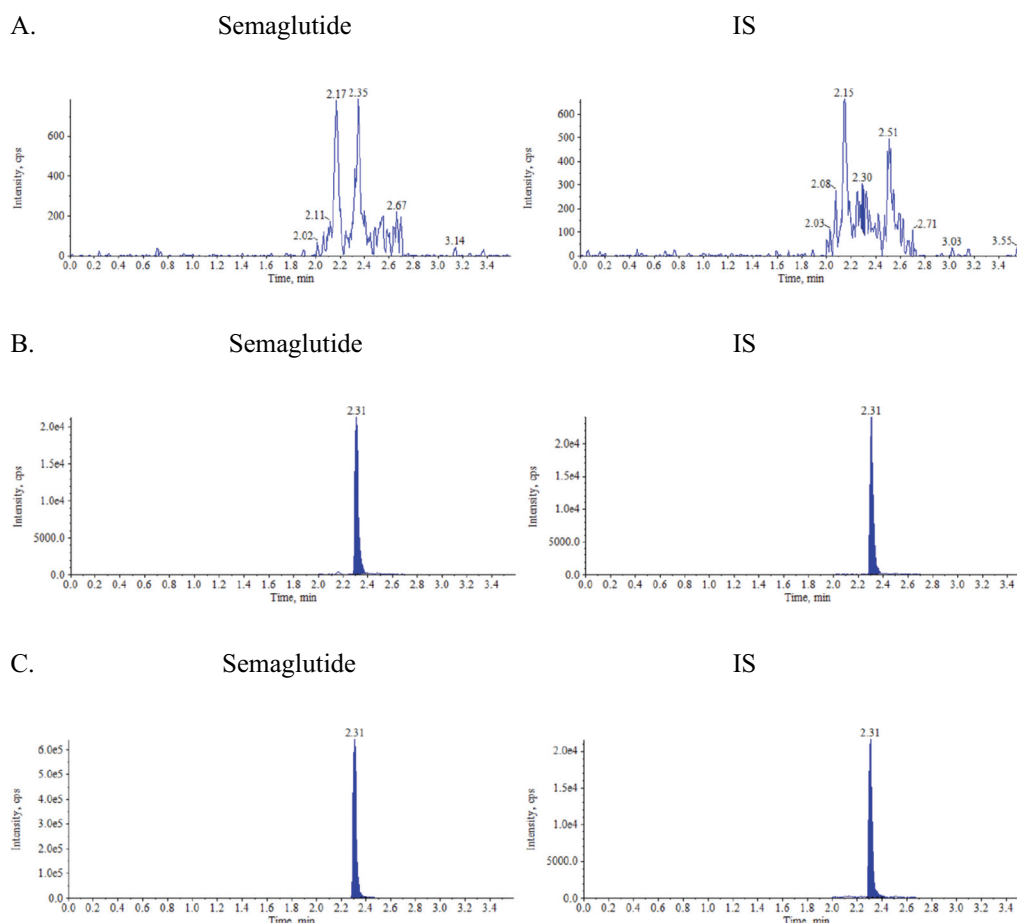


Figure 2. Representative MRM chromatograms of semaglutide and internal standard. (A) Matrix blank, (B) LLOQ standard, (C) ULOQ standard.

Table 2. Standard curve results.

	STD-5.00	STD-10.0	STD-20.0	STD-40.0	STD-80.0	STD-120	STD-180	STD-200
	5.00	10.0	20.0	40.0	80.0	120	180	200
Batch ID	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL
1	4.97	9.71	21.9	39.8	79.8	118	177	197
2	4.92	10.3	*14.4	42.1	75.4	117	185	198
3	4.90	10.4	20.1	39.5	75.7	121	190	194
4	4.99	9.76	21.2	39.8	83.1	117	181	191
5	4.97	9.96	20.3	41.8	78.5	119	181	195
6	4.84	10.4	21.5	38.0	81.7	123	172	192
7	4.96	10.1	19.7	41.8	80.4	121	177	194
8	5.17	9.28	19.9	39.6	86.4	120	173	201
Mean	4.97	10.0	20.7	40.3	80.1	120	180	195
SD	0.0961	0.393	0.864	1.45	3.69	2.14	6.05	3.28
% CV	1.9	3.9	4.2	3.6	4.6	1.8	3.4	1.7
% Accuracy	99.4	100.0	103.5	100.8	100.1	100.0	100.0	97.5
n	8	8	7	8	8	8	8	8

*Failed to meet acceptance criteria and excluded from the curve and summary statistics.

Table 3. Intra- and inter-batch accuracy and precision in QC measurements in human plasma.

	LLOQ-QC		LQC		MQC		HQC	% bias
	5.00	% bias	15.0	% bias	60.0	% bias	150	% bias
Batch ID	ng/mL		ng/mL		ng/mL		ng/mL	
1	5.40	8.0	14.2	-5.3	58.1	-3.2	133	-11.3
	5.51	10.2	15.0	0.0	59.4	-1.0	137	-8.7
	5.72	14.4	14.3	-4.7	59.3	-1.2	137	-8.7
	5.78	15.6	15.5	3.3	54.5	-9.2	140	-6.7
	5.45	9.0	15.0	0.0	56.2	-6.3	144	-4.0
	4.93	-1.4	14.4	-4.0	55.6	-7.3	145	-3.3
Intra-run Mean	5.47		14.7		57.2		139	
Intra-run SD	0.302		0.513		2.04		4.59	
Intra-run % CV	5.5		3.5		3.6		3.3	
Intra-run % Accuracy	109.4		98.0		95.3		92.7	
n	6		6		6		6	
2	3.68	-26.4	15.0	0.0	57.9	-3.5	146	-2.7
	5.02	0.4	14.5	-3.3	59.5	-0.8	162	8.0
	4.80	-4.0	14.6	-2.7	56.9	-5.2	163	8.7
	4.65	-7.0	15.1	0.7	59.8	-0.3	168	12.0
	4.88	-2.4	15.2	1.3	63.7	6.2	161	7.3
	4.77	-4.6	15.1	0.7	65.1	8.5	151	0.7
Intra-run Mean	4.63		14.9		60.5		159	
Intra-run SD	0.483		0.293		3.24		8.26	
Intra-run % CV	10.4		2.0		5.4		5.2	
Intra-run % Accuracy	92.6		99.3		100.8		106.0	
n	6		6		6		6	
3	4.94	-1.2	15.3	2.0	57.6	-4.0	137	-8.7
	4.65	-7.0	14.1	-6.0	56.5	-5.8	140	-6.7
	5.03	0.6	13.6	-9.3	60.5	0.8	146	-2.7
	4.22	-15.6	14.1	-6.0	57.1	-4.8	140	-6.7
	4.45	-11.0	14.1	-6.0	57.9	-3.5	129	-14.0
	5.67	13.4	15.2	1.3	59.3	-1.2	142	-5.3
Intra-run Mean	4.83		14.4		58.2		139	
Intra-run SD	0.511		0.687		1.49		5.73	
Intra-run % CV	10.6		4.8		2.6		4.1	
Intra-run % Accuracy	96.6		96.0		97.0		92.7	
n	6		6		6		6	
Inter-run Mean	4.98		14.7		58.6		146	
Inter-run SD	0.553		0.538		2.65		11.1	
Inter-run % CV	11.1		3.7		4.5		7.6	
Inter-run % Accuracy	99.6		98.0		97.7		97.3	
n	18		18		18		18	

3.1.6. Hemolysis effect

The mean accuracy and precision of the hemolysis LQC samples were 88.7% and 5.0%, respectively, and the mean accuracy and precision of the hemolysis HQC samples were 96.0% and 7.2%, respectively. The results demonstrated that there was no effect from hemolysis with this method.

3.1.7. Lipemic effect

The mean accuracy and precision of the lipemic effect LQC samples were 93.3% and 12.5%, respectively, and the mean accuracy and precision of the lipemic effect HQC samples were 104.0% and 2.4%, respectively. The results demonstrated that there was no effect from lipemia with this method.

3.1.16. Stock solution stability

The stability of semaglutide in a stock solution composed of 4% formic acid in methanol/water (90:10) was evaluated at room temperature for 6 hours and at 6 °C for 7 days. One aliquot of a stock standard solution was kept at room temperature for 6 hours, then diluted to a concentration for appropriate instrument response. Another stock solution was kept at 6 °C for 7 days and tested against a fresh stock solution in the same manner. An aliquot of a freshly prepared stock solution was diluted in the same manner. The difference between the mean instrument response (peak area ratio) of the test solutions and the freshly prepared solutions was 2.8% for the 6-hour stock solution and 8.5% for the 7-day stock solution. The results demonstrate the stock solution was stable on the benchtop (room temperature) for 6 hours and at 6 °C for 7 days.

4. Discussion and conclusion

The method validation described within resulted in a robust, highly sensitive method capable of quantifying semaglutide in human plasma using a stable labeled isotope internal standard, and capable of analyzing clinical samples across a wide concentration range (5 ng/mL to 1000 ng/mL). The method represents a relatively simple, protein-precipitation-based triple-quadrupole HPLC-MS/MS approach for this purpose. The validation of this method was in accordance with the current 2022 FDA M10 guideline [30] for method validation and sample analysis and met all the requirements for subsequent use in supporting clinical studies of semaglutide. This method demonstrates accuracy and precision, calibration curve linearity, selectivity, recovery, hemolysis effect, lipemic effect, matrix effect, carryover, dilution integrity, whole blood stability, reinjection reproducibility, and stability including long-term, benchtop, and freeze/thaw stability. Investigations of possible interference of concomitant medications (specificity) with the quantitation of semaglutide were not conducted as part of this validation, but a rationale provided, namely, of all of the possible concomitant drugs that was surmised to be administered, none had a Q1 mass that was within 20 daltons of semaglutide. Since clinical samples were not analyzed as part of this validation, incurred sample reanalysis could not be assessed, which is usually conducted following method validation and during sample analysis.

The development and validation of this method demonstrated, importantly, that protein precipitation was sufficient to extract semaglutide and its internal standard. Previously, the extraction of other GLP-1 peptide analogs [6,15,16,18,25,26,31] often used solid phase extraction (SPE) or supportive liquid extraction (SLE) sorbent plates, which are more time consuming, costly, and prone to potential variations in recovery [6,18,23,31–34]. Furthermore, the novel method established in this paper can be extended to the bioanalysis of other GLP-1 agonist peptide-based therapeutics with similar structural and physiochemical properties. Currently, our laboratory is exploring the capabilities of the high-resolution 8600 ZenoTOF (Sciex) mass spectrometer for the evaluation of GLP-1 peptides, including semaglutide, and

comparing the associated sensitivity to that of the 6500+ QTRAP (Sciex) used for this and other GLP-1 peptide analyses.

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Author contributions

CRediT: **Christopher Tudan**: Project administration, Writing – original draft; **Joel Stickling**: Investigation, Methodology, Validation; **Emily Northwick Darden**: Validation; **Lauren Devine**: Writing – review & editing; **Shane Needham**: Supervision, Writing – review & editing.

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Ethical declaration

This study utilized commercially purchased human serum samples obtained from BioIVT. All serum samples were procured from established commercial suppliers who maintain strict ethical standards and regulatory compliance in accordance with international guidelines for human biological materials.

Data availability statement

Raw data were generated at Veloxity Labs, LLC. Derived data supporting the findings of this study are available from the corresponding author Christopher Tudan on request.

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