

## Comprehensive Workflow for the Quantification of Peptides and Proteins in Plasma: Semaglutide a Case Study

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### Abstract

Over the last two decades we have seen a significant increase in protein and peptide-based therapeutics, with “biologics” accounting for 21 of the 55 new medicines approved in 2023, most notably the glucagon-like peptide-1 (GLP-1) receptor agonist semaglutide. These protein and peptide therapies typically show high potency and specificity they also have a long pharmacokinetic (PK) half-life resulting in nmol/L or lower concentration levels in the circulatory system requiring a high sensitivity bioanalytical assay to accurately define the compound PK. The development of a suitable LC-MS/MS method for the quantification of proteins and peptides in biofluids is complicated by the formation of precursor ions with multiple charge states and the large number of possible product ions to be evaluated. Here we demonstrate the use of an automated workflow to select, optimize and compare peptide MRM transitions via MassLynx™-Skyline Interface for the high sensitivity quantification of semaglutide in human plasma using the Xevo™ TQ Absolute Mass Spectrometer coupled with an ACQUITY™ Premier UPLC™.

### Benefits

- Xevo TQ Absolute Mass Spectrometer enabled high sensitivity quantification of semaglutide at sub ng/mL levels (LLOQ of 0.2 ng/mL) enabling accurate determination of PK elimination phase
  - Oasis™ MAX μElution mixed-mode SPE plates delivers high selectivity for isolation of semaglutide from
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human plasma enabling analyte concentration, cleaner extracts and improved sample recovery

- MaxPeak™ High Performance Surfaces (HPS): Mitigates non-specific binding facilitating increased sensitivity through high peptide recovery and improved peak shapes with HPS in Waters™ QuanRecovery™ Plates, Premier columns, and ACQUITY Premier UPLC
  - The integrated MassLynx-Skyline Interface (MSI) provides a workflow for simplifying MRM method development for peptides, eliminating the need to manually evaluate a large number of potential MRM transitions
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## Introduction

The development of therapeutic peptides in drug discovery has significantly progressed over the past decade and now account for a significant proportion of the pharmaceutical market.<sup>1,2</sup> Amongst the notable advancements in peptide therapeutics is the Glucagon-like peptide-1 (GLP-1) receptor agonist semaglutide, Figure 1. GLP-1 is an incretin hormone with metabolic effects including the stimulation of insulin secretion in response to glucose.<sup>3</sup> GLP-1 agonists, are routinely used in the treatment of type 2 diabetes to ensure glycaemic control and, more recently, for weight management/obesity.<sup>3</sup> These GLP-1 receptor agonists are typically dosed once per week and exhibit long half-lives with circulatory concentrations in the low nmol/L range, thus, to accurately define the pharmacokinetics (PK) of these compounds it is critical to develop sensitive and robust bioanalytical assays to quantify these drugs and their metabolites at very low levels in blood extracts. Previous clinical studies have shown that 20 days after a 1 mg intravenous administration, the concentration of semaglutide had reduced to 0.5 nmol/L with systemic clearance of 0.0348 L/h. Therefore, to accurately determine the PK elimination phase of semaglutide, a high sensitivity, high specificity bioanalytical assay is required in the sub ng/mL range.

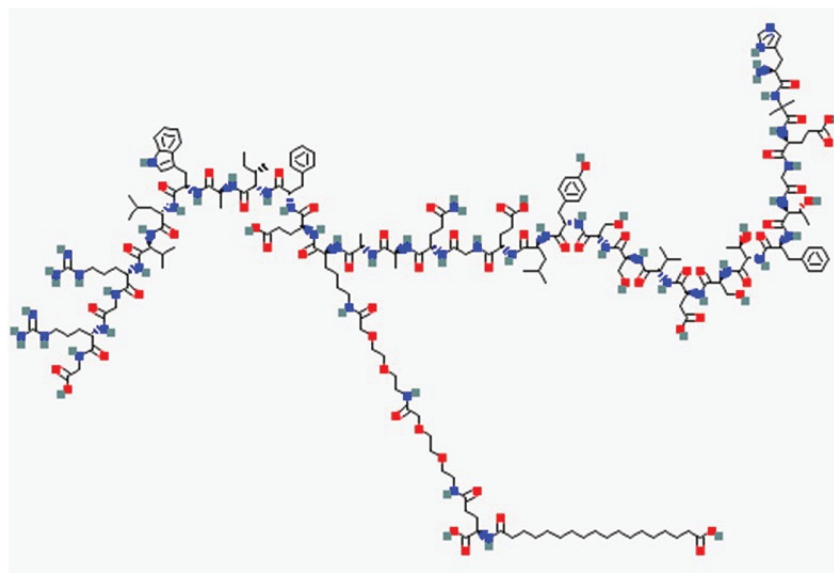


Figure 1. Structure of Semaglutide.

The sensitivity and specificity of LC-MS/MS is well suited to the quantification of low systemic concentration peptides like semaglutide. The development of bioanalytical assays for peptides, however, presents several challenges, namely MS method development, eliminating non-specific binding and management of chromatographic peak shape as peptides are well known to exhibit poor recovery and chromatographic peak shape due to non-specific binding to consumables and chromatographic components. The selection of the optimal MRM transitions for peptides is confounded by the numerous permutations of precursor and product ions which is exacerbated by the fact that peptides form multiple charge state precursor ions. Evaluating all these potential combinations of charge state/precursor ion/product ion/collision energy is a daunting task requiring the evaluation of multiple MRM transitions. Without tailored technologies and software, method development becomes a time-consuming task and presents risk to successful and efficient assay development.

To address these challenges of method development, optimization and robust routine operation, Waters have developed a series of analytical tools to simplify peptide quantitative bioanalysis. The MassLynx-Skyline Interface (MSI) was developed to simplify MS/MS optimization by facilitating the in-silico fragmentation of a peptide and automatic generation of precursor-product ion combinations for evaluation, eliminating the potential for manual transcription errors. The MaxPeak ACQUITY UPLC columns and vials and ACQUITY Premier UPLC chromatography system have been specifically designed with high performance surface technologies to mitigate and eliminate non-specific analyte binding to metal and glass surfaces. This application note describes a comprehensive workflow for the quantitative analysis of the GLP-1 peptide semaglutide by UPLC-

MS/MS, using the MassLynx-Skyline interface and ACQUITY Premier/MaxPeak technologies to deliver LLOQ of less than 0.2 ng/mL in human plasma.

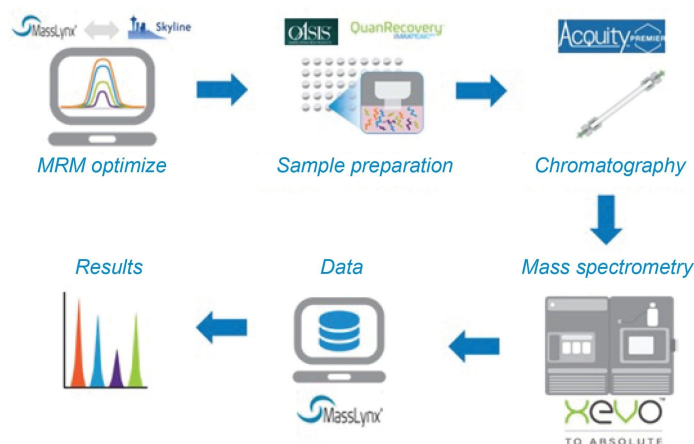


Figure 2. Schematic of the Optimised Comprehensive workflow for the bioanalytical analysis of peptides by LC-MS/MS.

## Experimental

A calibration curve was prepared in human plasma by spiking with semaglutide (AstaTech Inc.) at seven concentrations between 0.2 to 100 ng/mL. In addition, five replicate Quality Control (QC) standards were prepared in spiked human plasma at each of four concentrations (0.2, 0.3, 7, and 80 ng/mL). Standards and QCs (200  $\mu$ L) were extracted by addition of methanol (2:1 methanol: plasma) containing Liraglutide as an internal standard (in the absence of a stable label isotope version of semaglutide) (final concentration 0.5 ng/mL), vortexed for 1 minute and centrifuged at 14,000 *rcf* for 10 minutes. The extracts were subject to solid phase extraction (SPE) clean up using Oasis MAX, mixed mode polymeric SPE 96-well  $\mu$ Elution plate (p/n: [186001829 < https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186001829-oasis-max-96-well--elution-plate-2-mg-sorbent-per-well-30--m-1-p.html >](https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186001829-oasis-max-96-well--elution-plate-2-mg-sorbent-per-well-30--m-1-p.html) ). The final extract was eluted into Waters QuanRecovery plates to minimize non-specific binding and maximize peptide recovery.

Semaglutide quantification was performed on an ACQUITY Premier UPLC System, with separations performed on 2.1 x 50 mm 1.7  $\mu$ m ACQUITY Premier Peptide CSH™ C<sub>18</sub> 130 Å Column operated at 65 °C and a flow rate of 0.4 mL/min. Following an initial hold of 0.3 minutes at 30% B the column was eluted with a linear

reversed-phase gradient 30–65% B over the following 4.7 minutes, followed by a 2 minutes wash at 95% B, the column was then re-equilibrated at 30% B for 2 minutes prior to the next injection.

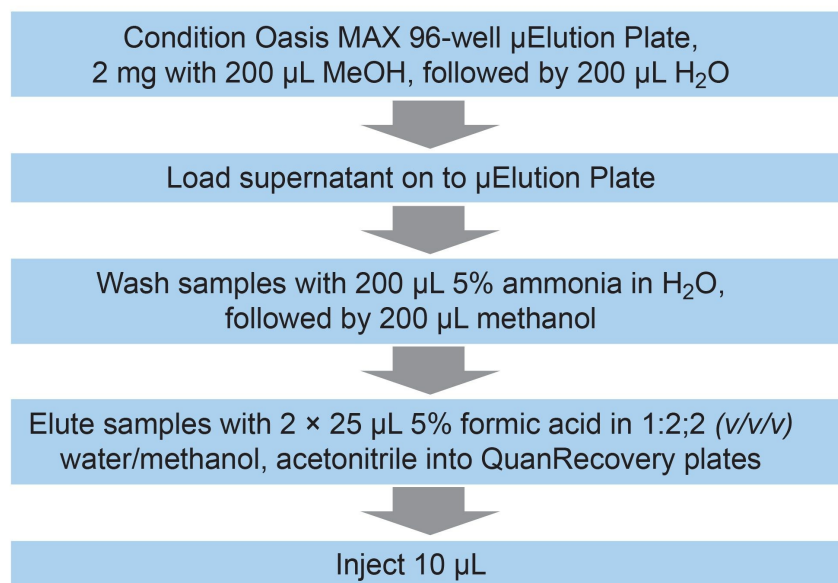


Figure 3. MRM transitions for semaglutide were optimized using the MassLynx Skyline Interface (MSI) tool.<sup>4</sup> The peptide sequence for semaglutide was input into Skyline and modified to include the adjusted mass for the non-standard amino acid 2-aminoisobutyric acid and the fatty acid side chain. The MSI automated and simplified the development of the MRM method to firstly determine the peptides retention time and then select the most sensitive MRMs and optimize the collision energies. Transitions were further fine-tuned by injection and manual data review.

## LC Conditions

LC system:	ACQUITY Premier UPLC
Vials:	QuanRecovery 96 well plates (p/n: 186009184)
Column(s):	ACQUITY Premier Peptide CSH C <sub>18</sub>

Column, 130 Å, 1.7 µm, 2.1 x 50 mm  
(p/n: 186009460)

Column temperature: 65 °C

Sample temperature: 8 °C

Injection volume: 10 µL

Flow rate: 0.4 mL/min

Mobile phase A: 0.1% formic acid (aq)

Mobile phase B: 0.1% formic acid in acetonitrile

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.4	70	30	Initial
0.3	0.4	70	30	6
5.0	0.4	35	65	6
5.1	0.4	5	95	6
7.1	0.4	5	95	6
7.2	0.4	70	30	6
10.0	0.4	70	30	6

## MS Conditions

MS system: Xevo TQ Absolute

Ionization mode: ES+

Capillary voltage: 2.0 kV

Desolvation temperature: 600 °C

Desolvation gas flow: 1000 L/Hr

## Peptide Table

Peptide	MRM	Cone (V)	Collision (v)
Semaglutide	1029.25>1238	30	30
Liraglutide	938.7>1064.3	30	22

## Data Management

Chromatography and MS software: MassLynx v 4.2

Informatics: MassLynx-Skyline Interface (MSI)

TargetLynx™ v 4.2

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## Results and Discussion

The accurate and reliable quantification of drugs and their metabolites in biological fluids requires sensitive, robust assays. LC-MS/MS has become established as the technology of choice for small molecule discovery and development bioanalysis, due to its sensitivity, specificity, and speed of method development. The quantification of peptides using LC-MS/MS for bioanalysis assays requires the development of solid phase extraction (SPE) and chromatography method to separate the peptide of interest from the endogenous components in a biofluid and a selective MRM MS method (the workflow employed in this study is shown in Figure 2).

Unlike small molecules, which by in large generate singly charged precursor ions in electrospray MS, peptides typically give rise to several multiply charged precursor ions. The formation of several multiply charged ions (+3, +4, +5, +6 *etc.*). Combined with the generation of multiple fragment ions and the requirement to select the best cone voltage and collision energy combination results in a large number of MRM transitions and energy combinations which all need to be evaluated and compared. This is a time consuming and laborious process requiring the acquisition and comparison of potentially hundreds of MRM transitions. To simplify the MRM data

acquisition and comparison process, we employed the MSI tool. The peptide sequence for semaglutide was input into the Skyline software, including a modification adjusted mass for the non-standard amino acid 2-aminoisobutyric acid and the fatty acid side chain, which performed in silico fragmentation to create a list of potential precursor and product ion combinations. This list was transferred to MassLynx where a range of collision energies was investigated for each MRM transition. The resulting raw data was then automatically transferred back to Skyline for evaluation and selection of the optimal MRM transition. The MSI is shown in Figure 4 for semaglutide MRM optimization. A total of 60 precursor ion – product ion – collision energy – cone voltage combinations were evaluated with the optimal transition determined to be ESI +ve 1029.25 → 1238 which corresponds to the  $[M-H]^{4+}$  ion.

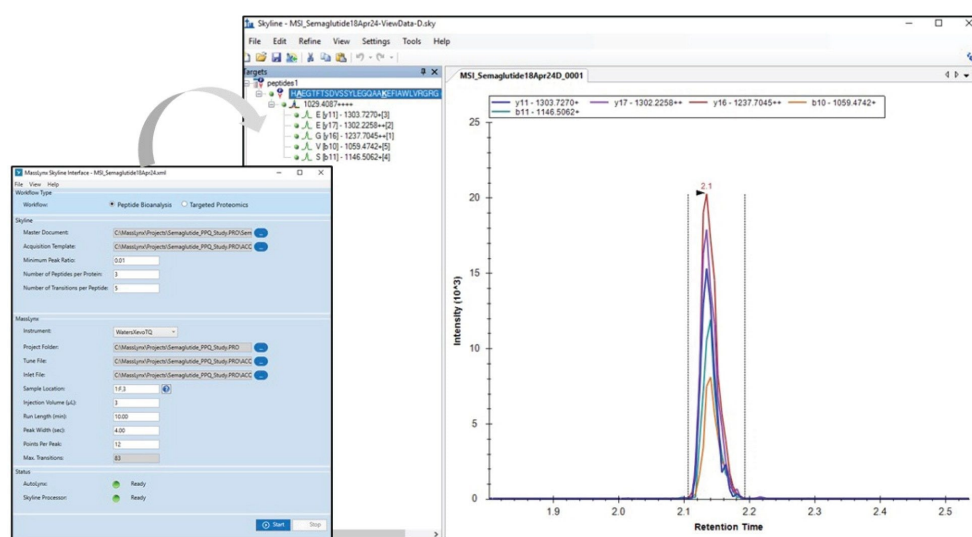


Figure 4. MSI used to optimize MRM transitions for the quantitative analysis of semaglutide.

Both the liquid chromatography system and columns were constructed using High Performance Surfaces (HPS) which prevent non-specific binding of metal sensitive analytes such as peptides in to commonly present metal components in the fluidic path, column as well as the plastic surfaces of the SPE recovery plate.<sup>5</sup> The synthetic peptide GLP-1 receptor agonist eluted from the chromatography column with a retention time of  $t_R=2.18$  min, Figure 5. The optimized method demonstrated excellent peak shape for semaglutide with no evidence of non-specific binding impacting chromatographic performance and no observable carryover. The method, based on SPE, was found to be linear ( $R^2=0.9985$ ) over the calibration range 0.2–100 ng/mL using 1/x weighting, Figure 7. The signal to noise value (S/N) for the 0.2 ng/mL calibrator was greater than 5:1 when compared to a blank plasma sample extract, Figure 6, indicating that there was no detectable plasma matrix interference which



could compromise assay specificity or limit of quantification.

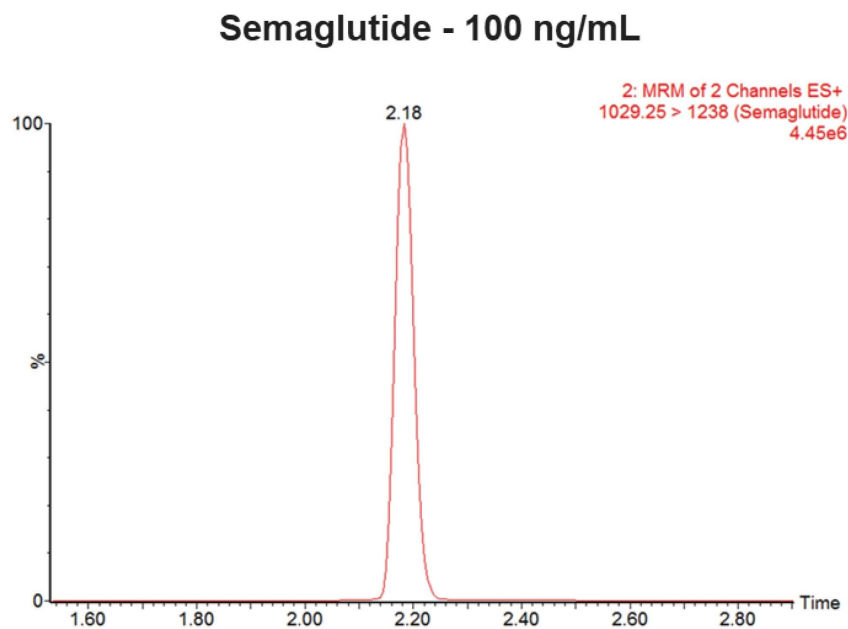


Figure 5. UPLC-MS/MS chromatogram showing semaglutide at 100 ng/mL.

Compound name: Semaglutide  
Correlation coefficient:  $r = 0.999245$ ,  $r^2 = 0.998491$   
Calibration curve:  $0.336476 * x + 0.0608956$   
Response type: Internal Std ( Ref 2 ), Area \* ( IS Conc. / IS Area )  
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

$R^2 = 0.998$

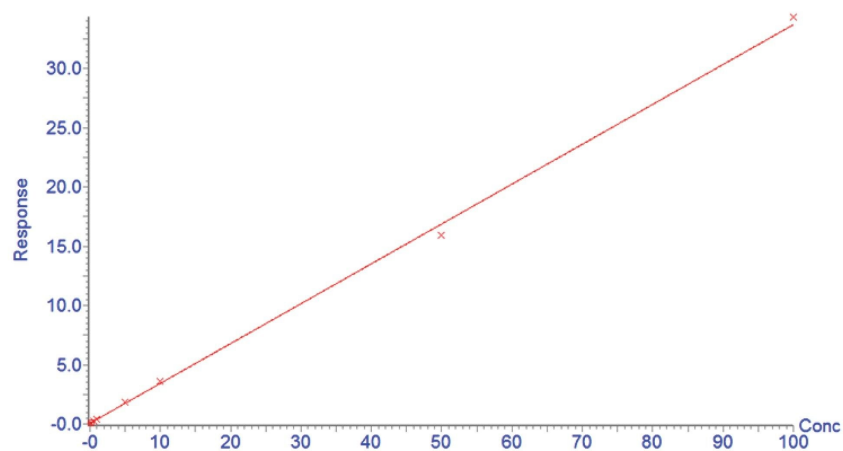


Figure 6. Calibration curve for semaglutide. An LLOQ of 0.2 ng/mL and linearity between 0.2 ng/mL and 100 ng/mL was achieved.

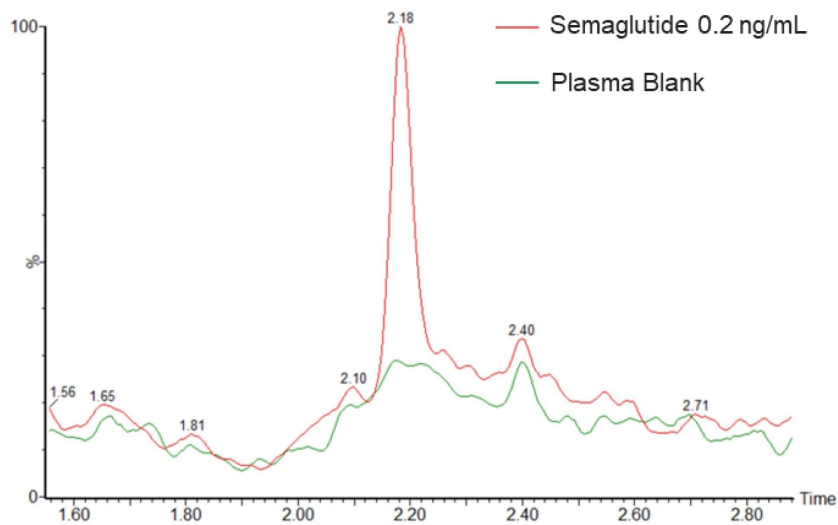


Figure 7. UPLC-MS/MS chromatogram showing semaglutide at LLOQ (0.2 ng/mL) overlaid with a plasma blank injection.

The repeatability and accuracy of quantification was assessed by the replicate preparation of QC samples which were interspersed throughout the sample analysis. The %CV and accuracy were <10.63 and >90% respectively and well within acceptable parameters for a discovery “fit for purpose” bioanalysis assay.

Sample replicate	LLQC (0.2 ng/mL)	LQC (0.3 ng/mL)	MQC (7 ng/mL)	HQC (80 ng/mL)
1	0.170	0.219	6.12	78.5
2	0.183	0.273	7.37	67.7
3	0.186	0.305	7.34	68.3
4	0.189	0.287	6.20	74.6
5	0.184	0.268	7.50	75.8
<b>Average conc. (ng/mL)</b>	0.182	0.270	6.91	73.0
<b>Std. dev</b>	0.007	0.029	0.611	4.28
<b>% CV</b>	<b>3.58</b>	<b>10.6</b>	<b>8.85</b>	<b>5.87</b>
<b>Average accuracy (%)</b>	91.2	90.1	98.7	91.2

Table 1. Repeatability and accuracy for the quantitative analysis of semaglutide in human plasma.

## Conclusion

A sensitive and selective assay for the quantification of semaglutide in human plasma has been successfully developed using the Xevo TQ Absolute with the ACQUITY Premier UPLC and OASIS MAX SPE. The described workflow provides analytical sensitivity down to 0.2 ng/mL for semaglutide. Excellent linearity was observed across the calibration range of 0.2–100 ng/mL and no analyte carryover or interference was observed in the blank plasma samples. Repeatability of the method was <4% at the LLQC level and accuracy of quantification was >90%, well within acceptable limits for a discovery “fit for purpose” bioanalytical assay. The MassLynx–Skyline Interface was used to simplify the MS MRM method development process allowing for greater than 60 combinations of precursor ion, product ion, collision energy, and cone voltages to be rapidly evaluated and the optimal combination selected. The use of ACQUITY Premier UPLC chromatography systems, MaxPeak UPLC columns and QuanRecovery plates mitigated analyte absorption to the metal and plastic surfaces of the chromatography system/columns and sample plates, resulting in symmetrical LC peak shapes

and minimal analyte loss.

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