COMPREHENSIVE WORKFLOW FOR QUANTITATIVE BIOANALYSIS OF LARGE PEPTIDES AND PROTEINS: A CASE STUDY OF THE GLP-1 RECEPTOR AGONIST, SEMAGLUTIDE, IN PLASMA

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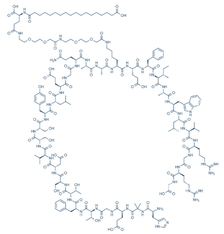
INTRODUCTION

Critical to advancing all medicines is the development of precise bioanalysis assays to determine compound PK, typically using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

Over the past decade, significant advances have propelled peptide drug development forward. Among the notable advancements is semaglutide, a high-profile 32 amino acid.glucagon-like-peptide-1 receptor agonist, regulatory approved for treatment of both type 2 diabetes and weight management/obesity.

Molecular Structure of GLP-1 receptor agonist semaglutide



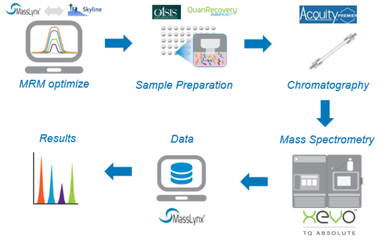
The sensitivity and selectivity of LC-MS/MS is well suited to the quantification of low systemic concentration of peptides like semaglutide. However, efficient development of peptide bioanalysis assays presents challenges namely:-

- Selection of the optimal MRM transitions among countless permutations of parent ion, fragments and charge states
- Poor chromatographic peak shape and sample preparation recovery due to non-specific binding
- Low solubility of hydrophobic peptides

Without tailored technologies and software method development becomes time-consuming and presents risk to developing fully optimized assay in sensitivity, accuracy, and robustness.

This case study presents a comprehensive workflow to address these method development hurdles using the example of the quantitative LC-MS/MS analysis of semaglutide in human plasma

COMPREHENSIVE WORKFLOW



Key Features

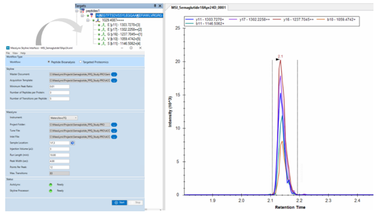
- Rapid *in silico* MRM optimisation using Skyline (MacCoss Lab) accessible via integrated Waters™ MassLynx™ Software interface
- High Performance Surface technologies (HPS) to minimize loss and poor recovery of peptides
  - QuanRecovery™ low bind 96 well plates and vials
  - ACQUITY™ Premier Column technology
  - ACQUITY Premier UPLC™ system
- High performance Waters Xevo™ TQ Absolute tandem mass spectrometer and MassLynx software

LC-MS/MS Conditions	
LC –MS/MS System	Waters ACQUITY Premier UPLC with Xevo TQ-Absolute Tandem MS
ESI +ve Capillary	2.0 kV
Desolvation temperature	650°C
Semaglutide MRM transition	1029.3 to 1238.0 Da
Cone Voltage	30 V
Collision Energy	30 eV
Column	Waters ACQUITY Premier Peptide CSH™ C18 130Å, 1.7µm, 2.1mm x 50mm
Column Temperature	65 °C
Injection volume	10µL
Flow Rate	0.4mL/min
Mobile Phase A	0.1% formic acid (aq)
Mobile Phase B	0.1% formic acid in acetonitrile

MRM OPTIMIZATION VIA MASSLYNX-SKYLINE INTERFACE

- The *in silico* MRM optimisation software Skyline enabled the rapid identification of the $[M+4H]^{4+}$ precursor and doubly charged product ion, (corresponding to the fragmentation of the y16 amino acid), as optimum MRM transition for semaglutide peptide. (Ref 1)
- MRM collision energy scouting data, generated in MassLynx was efficiently visualized for refinement in Skyline.
- Transitions were further fine turned by injection for optimum sensitivity.
- The editable nature of the Skyline methodology enabled inclusion of the nonstandard amino acid 2-aminoisobutyric acid and fatty acid side chain of semaglutide.

The Skyline/ MassLynx I MRM optimisation workflow



SAMPLE PREPARATION

- To prepare the calibration curve, human plasma was spiked with semaglutide (AstaTech Inc,) at 7 concentrations between 0.2 to 100ng/mL.
- 5 replicate Quality Control standards were prepared at each of 4 concentrations (0.2, 0.3,7 and 80ng/mL)
- Prior to SPE, extraction, standards and QCs were subject to pre clean-up by protein precipitation in methanol (2:1 methanol: plasma) containing Liraglutide internal standard (final concentration 0.5ngmL).
- All standards and QCs were subject to solid phase extraction clean up using Oasis™MAX, mixed mode polymeric SPE 96-well µElution plate, eluting finale extract into Waters QuanRecovery plates to minimize non-specific binding.
- Samples were injected onto the LC-MS/MS system

Step by Step SPE Extraction Method

Condition Oasis MAX 96-well µElution Plate, 2 mg with 200 µL MeOH, followed by 200 µL H_2O

↓

Load supernatant on to µElution Plate

↓

Wash samples with 200 µL 5 % ammonia in H_2O, followed by 200 µL methanol

↓

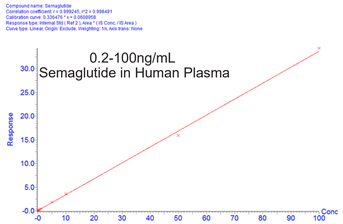
Elute samples with 2 x 25 µL 5% formic acid in 1:2:2 (*v/v/v*) water/methanol/acetonitrile into QuanRecovery plates

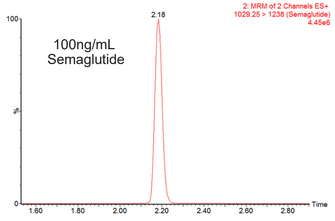
↓

Inject 10 µL

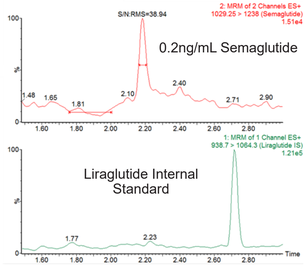
RESULTS AND DISCUSSION

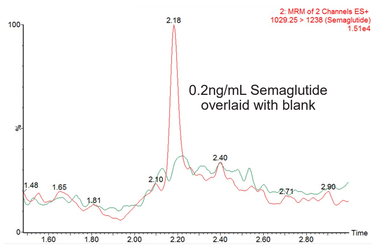
The optimized method demonstrated excellent sensitivity & linearity across the calibration range 0.2 to 100ng/mL plasma with coefficient of determination R2 = 0.998. Excellent chromatographic peak shape was observed with no evidence of non specific binding impacting chromatographic performance,





The assay limit of quantification was determined to be 0.2ng/mL with signal to noise (RMS) >30, meeting sensitivity requirement for therapeutic purposes of semaglutides. There was no observable carryover in the blanks.





Repeatability and accuracy of the quantitation method was evaluated through the analysis of replicate preparations of QC standards interspersed through the sample analysis. CV and accuracy were <10.63% and >90% respectively and well within acceptable parameters for bioanalysis assay validation, indicating robust and reliable methodology appropriate for bioanalysis studies.

	Calculated Concentration (ng/mL)			
Sample Replicate	**LLQC (0.2 ng/mL)**	**LQC (0.3 ng/mL)**	**MQC (7 ng/mL)**	**HQC (80 ng/mL)**
1	0.17	0.22	6.12	78.52
2	0.18	0.27	7.37	67.66
3	0.19	0.30	7.34	68.31
4	0.19	0.29	6.20	74.57
5	0.18	0.27	7.50	75.84
Average Conc. (ng/mL)	0.18	0.27	6.91	72.98
Std. Dev	0.01	0.03	0.61	4.28
% CV	**3.58**	**10.63**	**8.85**	**5.87**
Average Accuracy (%)	91.20	90.13	98.67	91.23

CONCLUSION

Developing bioanalytical assays for large peptides poses unique challenges including navigating the permutations of the MRM and MS parameter optimization

Hydrophobic peptides characteristically demonstrate poor solubility and often nonspecific binding, affecting analyte recovery and assay sensitivity

For semaglutide in human plasma, the optimized workflow demonstrated assay sensitivity of 0.2ng/mL, with excellent linearity spanning 0.2ng/mL to 100ng/mL and assay accuracy and precision well within acceptable limits of typical bioanalytical assays. There were no indication of non specific binding of semglutides observed in this assay

References:

1: MassLynx-Skyline Interface (MSI) – A New Automated Tool to Streamline MRM Method Development and Optimization for Large Molecule Quantification:Nikunj Tanna, Caitlin Dunning, Billy J. Molloy, Waters Corporation

2:SPE-LC/MS Bioanalytical Quantification of the Biotherapeutic Peptide, Semaglutide From Plasm: Mary Trudeau, Angela Scumaci, Waters Corporation

The authors declare no competing financial interest.

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