

Nota applicativa

Developing a Quantitative Surrogate Peptide Assay – Peptide mapping through MRM Optimization for Measuring Dulaglutide in a Rat PK Study

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Abstract

Development of fusion protein therapeutics, such as dulaglutide (TRULICITY™), is rising and there is a need for universal workflows which can be used to rapidly develop quantitative LC-MS/MS assays. The work herein describes a complete workflow for assay development of Fc containing therapeutics. Peptide mapping experiments were used to characterize and identify appropriate peptides for quantification. This was followed by optimization of MRMs via an automated MassLynx Skyline Interface workflow. Samples were prepared for analysis using a generic immunoaffinity capture method, a fast and reproducible digestion workflow, and selective peptide SPE. Achieving lower limits of quantification of 1 ng/mL dulaglutide, this assay was successfully deployed in the analysis of a rat PK study.

Benefits

- Peptide mapping using the Xevo G2-XS for the identification and selection of unique surrogate peptides
- MassLynx Skyline Interface (MSI) to rapidly select and optimize peptide MRM transitions
- High performance separation using BEH particle and ACQUITY UPLC I-Class Plus technologies
- High sensitivity quantification of dulaglutide, using a Xevo TQ-XS Mass Spectrometer

Introduction

Workflows supporting the rapid development of quantitative LC-MS/MS based assays for next generation therapeutics, such as fusion proteins, are essential. Although LC-MS/MS assays are ubiquitous in discovery and development labs, there is still room for improvement in the method development process in terms of rapidly characterizing and selecting peptides of interest for optimization. Here, we describe an overall process for rapid protein confirmation, peptide identification, selection, and optimization for dulaglutide, a fusion protein of a GLP-1 analogue and IgG4 Fc using a Xevo G2-XS QToF (for rapid characterization) and transfer to a triple quadrupole Xevo TQ-XS system (for rapid quantitative analysis). A second companion application note (720006823EN <<https://www.waters.com/webassets/cms/library/docs/720006823en.pdf>>) was published which discusses the final LC-MS/MS method and bioanalytical assay merits in more detail.

The workflow to design an assay ideally consists of the following steps (Figure 1);

- 1) HRMS confirmation and characterization
 - a. peptide mapping experiment for confirmation
 - b. determination of peptides suitable for quantitative monitoring (stability, deamidation, modification levels)
- 2) Triple Quadrupole optimization and analysis
 - a. rapid selection and optimization of MRM transitions
 - b. optimization of sample preparation methods (described in detail, application note [720006823EN < https://www.waters.com/webassets/cms/library/docs/720006823en.pdf>](https://www.waters.com/webassets/cms/library/docs/720006823en.pdf))¹

This application note shows the steps required to rapidly characterize, confirm the sequence, and apply this information to build a method for the analysis of dulaglutide in a rat pharmacokinetic (PK) study. HRMS platforms also have the capability to run the assay itself, however, this workflow describes the more conventional route of running the assay on a triple quadrupole platform for maximum throughput.² Lower limits of quantification of 1 ng/mL (0.02 M) were achieved, enabling high sensitivity quantitation of dulaglutide.

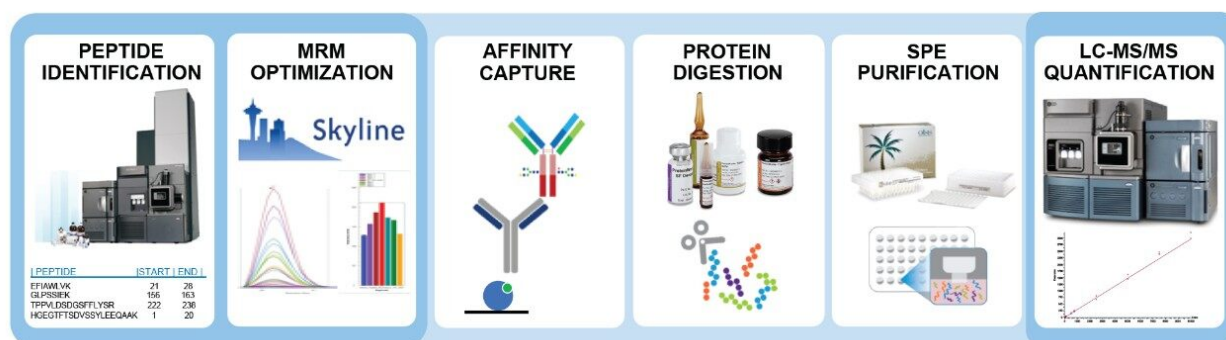


Figure 1. Quantitative assay development workflow utilizing peptide mapping, MassLynx Skyline Interface (MSI) MRM optimization, sensitive and selective sample preparation techniques, and Xevo TQ-XS for LC-MS/MS quantification.

Experimental

Sample Preparation

Preparation of Samples for Peptide Mapping and Skyline Optimization

Neat dilutions of dulaglutide were digested via the ProteinWorks Auto eXpress Low Volume Protocol (p/n: [176004077 <https://www.waters.com/nextgen/in/en/shop/application-kits/176004077-proteinworks-auto-express-low-3-digest-kit.html>](https://www.waters.com/nextgen/in/en/shop/application-kits/176004077-proteinworks-auto-express-low-3-digest-kit.html)). Briefly, dulaglutide was diluted to 300 µg/mL (24 µg) and denatured with *Rapi*Gest SF Surfactant, followed by reduction with dithiothreitol and alkylation with iodoacetamide. Samples were digested with trypsin using a 1:10 trypsin:protein ratio (w/w) and quenched with formic acid. Peptide mapping using LC-MS^E analysis was performed by injecting 10 µL (~1.5 µg) of sample on to an ACQUITY UPLC I-Class PLUS and Xevo G2-XS QToF Mass Spectrometer System. MassLynx Skyline Interface MRM optimization experiments were performed using a Xevo TQ-XS Mass Spectrometer, 1 µL injected per run.

Preparation of Rat PK Samples

Six rats were dosed subcutaneously at 1 mg/kg with dulaglutide and blood was collected pre-dose and again at 2, 4, 7, 24, 48, 72, 96, 120, and 168 hours post-dose. Complete instructions for sample preparation are outlined in application note [720006823EN <https://www.waters.com/webassets/cms/library/docs/720006823en.pdf>](https://www.waters.com/webassets/cms/library/docs/720006823en.pdf) .

Peptide Mapping LC-MS/MS Method Conditions:

LC system:	ACQUITY UPLC I-Class PLUS (Fixed Loop)
Detection:	Xevo G2-XS QToF Mass Spectrometer, ESI+
Plates:	QuanRecovery with MaxPeak High Performance Surfaces 96-well plates
Column(s):	ACQUITY UPLC Peptide BEH C ₁₈ , 300 Å, 1.7 µm, 2.1 x 100 mm
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	0.2 mL/min

Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Gradient:	1–35% B in 50 minutes at 0.2 mL/min followed by column wash and equilibration
MS system:	Xevo G2-XS QTof Mass Spectrometer, ESI+
Ionization mode:	ESI+
Capillary voltage:	1.2 kV
Cone voltage:	40 V
Source temp.:	150 °C
Desolvation temp.:	350 °C
Cone gas flow:	20 L/Hr
Desolvation gas flow	600 L/Hr
MS software	UNIFI (v.1.9.4)
Informatics	UNIFI (v.1.9.4)

Skyline and PK Study LC-MS/MS Method Conditions:

For complete LC-MS/MS conditions, see application note [720006823EN](#) <
<https://www.waters.com/webassets/cms/library/docs/720006823en.pdf>> .

Results and Discussion

Peptide Mapping for the Identification of Quantitative Peptides

Peptides suitable for quantification can be identified via peptide mapping experiments. Downstream sample preparation includes digestion of dulaglutide via the ProteinWorks Auto eXpress Low Digest Kit. Although this kit is not intended for peptide mapping applications which typically use (higher cost) sequencing grade material, it allows us to identify which peptides will be liberated from the protein via tryptic digestion (using the intended sample preparation method) and to assess their behavior. Dulaglutide peptides were identified with 93% sequence coverage (Figure 2, Panel A) using these methods.

Peptide modifications and missed cleavages were easily identified. Peptide mapping experiments for dulaglutide identified 15 tryptic peptides ranging from 6–33 amino acids in length (identified peptides less than 6 amino acids were excluded from this list due to poor specificity and retention). From this list (Figure 2, Panel B), peptides were further filtered on the basis of amino acid length (8–25 residues) and percentage of identified modifications. One peptide with a high level of observed modifications, EFLAWLVK, remained in the list of filtered peptides due to its unique position in the Fc fusion protein. This peptide comes from the GLP-1 region of the protein, one of only 2 peptides from this region. This filtered list of peptides forms the basis for the next step of assay development, optimization of MRM transitions.

A Dulaglutide

Identified: 93%

1: 1 to 50	<u>H</u> GEGTFTSDV	<u>S</u> SYLEEQA <u>A</u> K	<u>E</u> FI <u>A</u> WL <u>V</u> KGG	<u>G</u> GGGG <u>S</u> GGGG	<u>S</u> GGGG <u>S</u> A <u>E</u> SK
1: 51 to 100	<u>Y</u> GPPC <u>P</u> PC <u>P</u> A	<u>P</u> E <u>A</u> AG <u>G</u> PS <u>V</u> E	<u>L</u> FPPK <u>P</u> K <u>D</u> TL	<u>M</u> ISRT <u>P</u> EV <u>T</u> C	<u>V</u> VVD <u>V</u> SO <u>E</u> DP
1: 101 to 150	<u>E</u> VQFN <u>W</u> Y <u>V</u> DG	<u>V</u> EVH <u>N</u> A <u>K</u> TK <u>P</u>	<u>R</u> EEQFN <u>S</u> TYR	<u>V</u> VS <u>V</u> LT <u>V</u> LV <u>L</u> HQ	<u>D</u> WLN <u>G</u> KEY <u>K</u> C
1: 151 to 200	<u>K</u> VS <u>N</u> K <u>G</u> LP <u>S</u> S	<u>T</u> E <u>K</u> T <u>I</u> SK <u>A</u> K <u>G</u>	<u>Q</u> PRE <u>P</u> OV <u>Y</u> TL	<u>P</u> PSQE <u>E</u> MT <u>K</u> N	<u>O</u> VSLT <u>C</u> LV <u>K</u> G
1: 201 to 250	<u>F</u> YPS <u>D</u> I <u>A</u> VE <u>W</u>	<u>E</u> SN <u>G</u> OP <u>E</u> NN <u>Y</u>	<u>K</u> TTP <u>P</u> VL <u>D</u> SD	<u>G</u> SFFLY <u>S</u> R <u>L</u> T	<u>V</u> DKSR <u>W</u> OE <u>G</u> N
1: 251 to 275	<u>V</u> FSC <u>S</u> VM <u>H</u> EA	<u>L</u> HN <u>H</u> Y <u>T</u> Q <u>K</u> SL	SLSLG		

B

Candidate peptides	Amino acid length	Identified modifications	Meets filter criteria?
AKGQPR	6		
LTVDKSR	7		
DTLMISR	7	9.8% Deamidation	
GLPSSIEK	8		✓
EFIAWLVK	8	4.6% Oxidation	✓
NQVSLTCLVK	10		✓
VVSVLTVLHQDWLNGK	16	9.9% Deamidation	
EPQVYTLPPSQEEMTK	16		✓
TTPPVLDSDGSFFLYSR	17		✓
HGEGTFTSDVSSYLEEQAAK	20	1.1% Deamidation	✓
GFYPSDIAVEWESNGQPENNYK	22	10.9% Deamidation	
GGGGGGGGSGGGGGGGGSAESK	22		
WQEGNVFSCSVMHEALHNHYTQK	23		✓
YGPPCPCPAPEAAGGPSVFLFPPKPK	27		
TPEVTCVVVDVSOEDPEVQFNWYVDGVEVHNAK	33		

Figure 2. 93% sequence coverage of dulaglutide achieved via peptide mapping experiments (Panel A). 15 peptides of suitable length for quantification consideration were identified and 7 peptides which meet filter criteria were identified (Panel B).

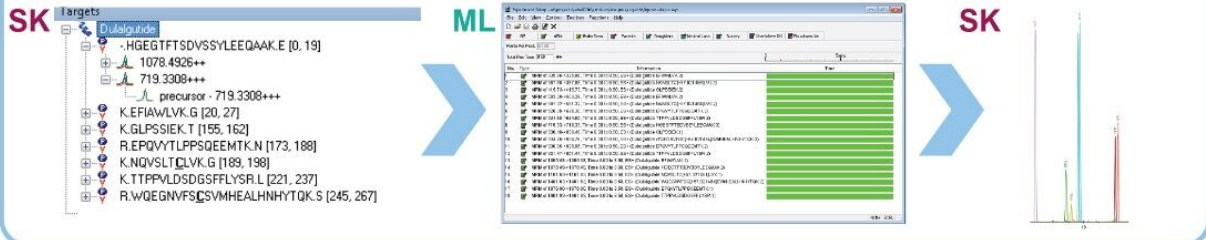
MassLynx Skyline Interface MRM Identification and Optimization

Skyline (MacCoss Labs, University of Washington), open access software, has been integrated with MassLynx software to provide a seamless workflow for MRM identification and optimization (collision energy, retention time scheduling, dwell time, etc.) and can save days to weeks of optimization and development time.³ MSI automates the multiple steps of acquisition method creation, data acquisition, and data review to

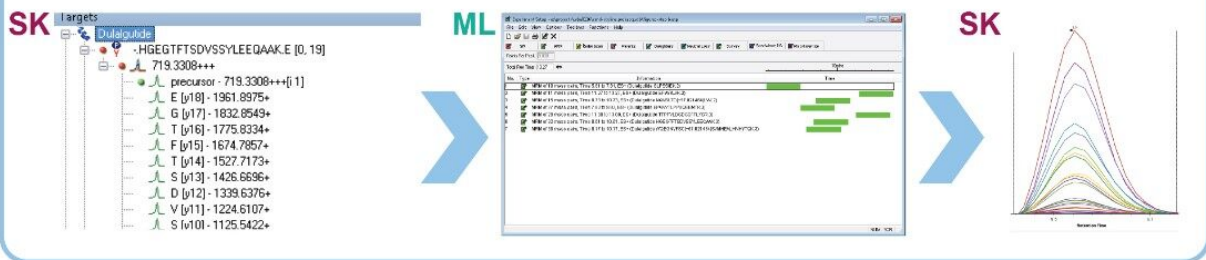
provide a final method with the most sensitive peptides per protein and the most sensitive transitions for each peptide. An example of the peptide bioanalysis workflow is shown in Figure 3.

SK = SKYLINE ML = MASSLYNX

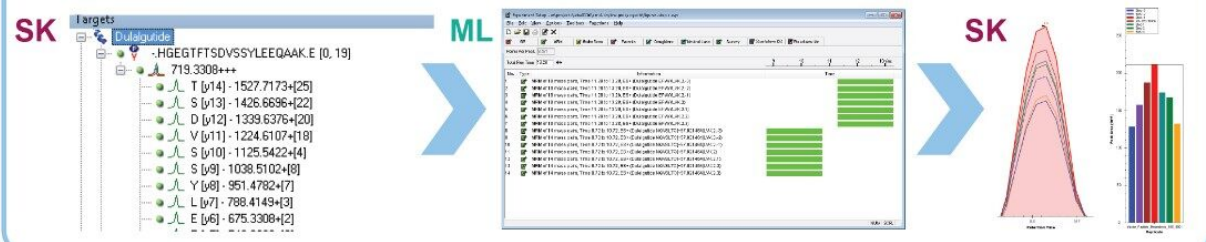
A RETENTION TIME ALIGNMENT AND PRECURSOR SELECTION



B MRM IDENTIFICATION



C COLLISION ENERGY OPTIMIZATION



D FINAL METHOD CREATION

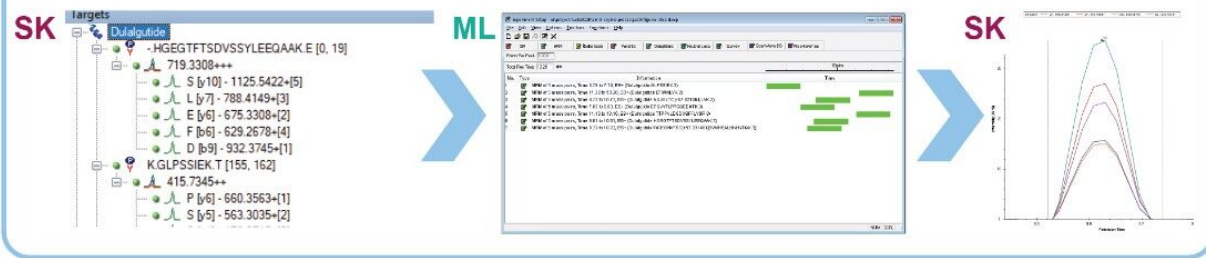


Figure 3. MassLynx Skyline Interface workflow for the identification and optimization of MRM transitions. Steps include: retention time and precursor m/z identification (Panel A), MRM identification (Panel B), MRM optimization for collision energy (Panel C), and final method creation using optimized settings (Panel D). Each import/export step is indicated by ML (MassLynx) or SK (Skyline) to indicate which software package is used.

Step A – Retention Time Alignment and Precursor Selection

- Candidate peptide sequences identified via peptide mapping are copied into a skyline master document provided with the download package of MSI. This master document contains appropriate settings for peptide MRM identification and optimization.
- Precursor m/z for each peptide are calculated via Skyline and are automatically exported into a MassLynx mass spectrometry method file (.exp).
- The digested protein of interest is injected for LC-MS/MS analysis using the exported MassLynx method. Results are then imported into the original Skyline document.
- The retention time of each peptide is identified with no manual intervention needed. The most intense precursor m/z for each peptide is then identified. These are used for the subsequent optimization steps.

Step B – MRM Identification

- A new MassLynx, time scheduled method file containing product ions is exported from Skyline.
- The digested protein is injected again for analysis and results are imported back into Skyline to identify observed MRM transitions.

Step C – MRM Optimization

- MRM transitions observed in step B will be indicated by a green light after data import. Transitions which were not observed will be indicated by a red light. Selecting each peptide will show the intensity of each MRM transition in one chromatogram for easy viewing. MRM transitions in the transition list are ranked according to intensity.
- All observed MRM transitions will then be optimized for collision energy. An appropriate number of MassLynx methods will be created automatically to accommodate the number of peptides and MRM transitions to be optimized in this step.
- The exported MassLynx methods contain 7 functions per peptide. Each function contains all MRM transitions observed in the previous step and collision energy will differ by 2 eV for each function. Another injection of the protein of interest is made and acquired data is imported into Skyline.

Step D – Final Method Creation

- Using the results acquired in the previous step, Skyline identifies the optimal collision energy value for each MRM transition and stores that information in the document.
- The document will be refined to remove all but the most intense MRM transitions.

- A final MassLynx method with the top MRM transitions and their optimized collision energies is exported from Skyline to use for further method development.

Following optimization of peptides via Skyline, MRM transitions must be assessed for sensitivity and selectivity in desired biological matrix. The most selective MRMs will generally have higher precursor and product m/z values.

Application of Workflow to a Rat PK Study

The workflow described here was applied to develop a sensitive assay to support the quantitative analysis of dulaglutide extracted from rat plasma. Rats were dosed with dulaglutide and samples were collected pre-dose and out to 168 hours post-dose in order to examine the pharmacokinetic (PK) properties of dulaglutide in rats. To gain an understanding of the entire dulaglutide protein, surrogate peptides were chosen from both the GLP-1 and Fc regions. The N-terminal peptide of GLP-1, HGEGTFTSDVSSYLEEQAAK (HGEG) was chosen as a surrogate for the intact fusion protein containing at least one copy of the GLP-1 sequence. From the Fc region of the protein, GLPSSIEK (GLPS) was chosen as a surrogate for the total Fc level.

The PK profile (Figure 4) demonstrates differential clearance of the HGEG and GLPS peptides. The half-life of the biotherapeutic was measured (using the HGEG peptide) to be 22.9 hours (calculated using PKSolver) and was consistent with that previously reported in literature.⁴⁻⁶ The half-life of the GLPS peptide was approximately 3 times longer, likely caused by observation of intact GLPS peptide and free GLPS from proteolytic cleavage in the GLP-1 region of dulaglutide in the plasma.⁷ It is necessary to understand the nature of the biotherapeutic in order to choose peptides judiciously and to gain a clear understanding of the entire protein behavior in PK studies.

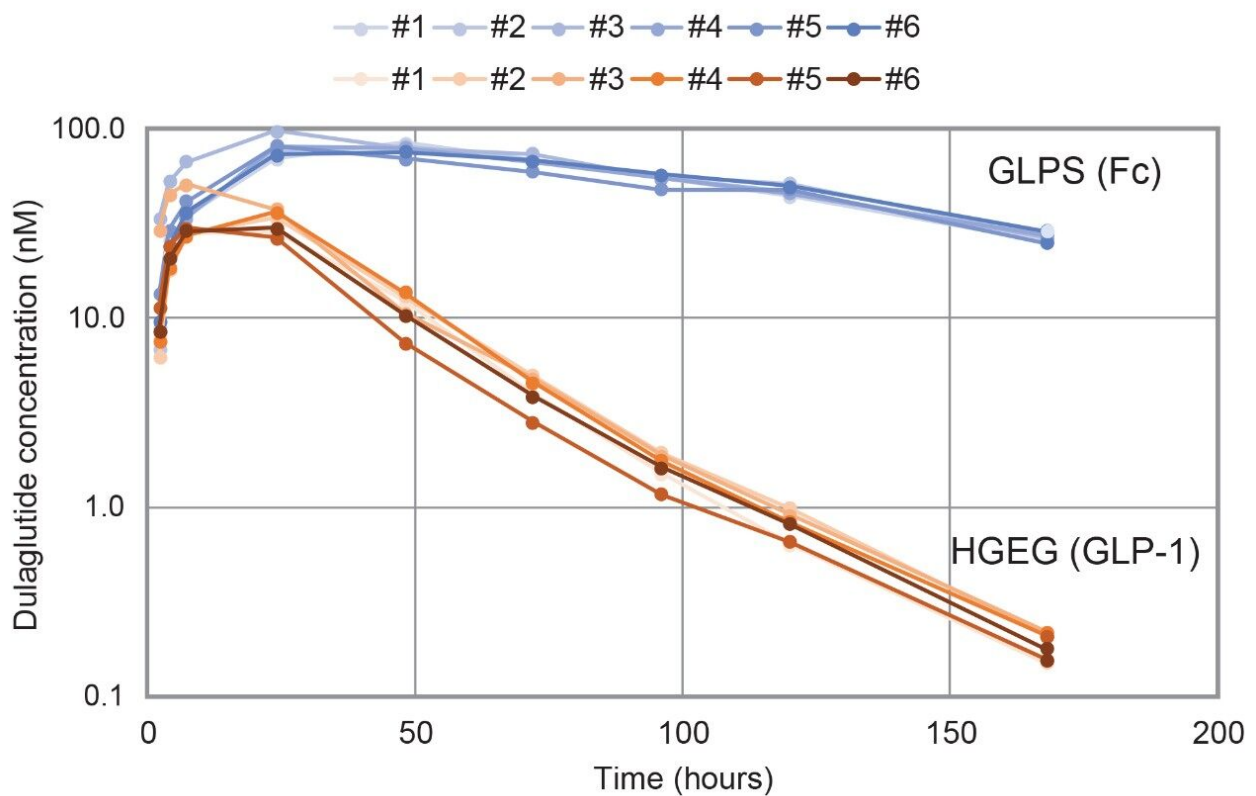


Figure 4. Pharmacokinetic profile of rats dosed with dulaglutide. The GLPS peptide served as a surrogate for the dulaglutide Fc region, and HGEG peptide for the GLP-1 region.

Linear, precise, and accurate quantification of dulaglutide was achieved using 50 μ L of rat plasma, which was immunopurified, digested, and SPE purified. Lower limits of quantification (LLOQs) of 1 ng/mL (0.02 nM) were achieved and calibration curves were linear ($r^2 > 0.99$) from 1–10,000 ng/mL (0.02–167.59 nM) using a $1/X^2$ linear fit (Table 1). QC levels were accurate within $\pm 15\%$ and CVs were $< 7\%$ and were consistent with standard bioanalytical method validation guidance criteria. QC performance statistics for all runs are highlighted in Table 2.

CALIBRATION CURVE STATISTICS				
Peptide	Curve (ng/mL)	Weighting	Linear fit (r ²)	% Accuracy range
GLPSSIEK	1-10,000	1/X ²	0.994	89.9-107.1
HGEGTFTSDVSSYLEEQAAK	1-10,000		0.991	86.2-108.3

Table 1. Linear dynamic range and standard curve statistics for dulaglutide peptides extracted from rat plasma.

Peptide	QC level	QC concentration (ng/mL)	QC STATISTICS		
			Mean (N=3) calculated QC concentration (ng/mL)	Mean (N=3) % accuracy	Mean (N=3) % CV
GLPS	LLOQ	3	2.6	85.9	0.8
	LQC	75	74.6	99.4	2.4
	MQC	750	783.6	104.5	0.3
	HQC	7500	8169.5	108.9	1.0
HGEG	LLOQ	3	3.0	100.7	5.9
	LQC	75	79.2	105.6	5.8
	MQC	750	800.9	106.8	7.1
	HQC	7500	8239.9	109.9	5.6

Table 2. QC quantitative performance for dulaglutide peptides extracted from rat plasma.

Conclusion

The work described here includes a complete workflow for the identification and optimization of surrogate peptides. This workflow has been applied to the development of a dulaglutide quantitative assay. Combined with advanced sample preparation techniques, rat PK samples were analyzed with high accuracy and precision.

- Peptide mapping experiments were used to identify and refine a list of surrogate peptides to be pursued for the quantification of dulaglutide

- MassLynx Skyline Interface software was implemented to identify and optimize MRM transitions for chosen surrogate peptides
- Dulaglutide was quantified in rat PK samples with high precision and accuracy over the range of 0.15–98.21 nM

References

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