

응용 자료

## Development of an Effective LC-MS/MS Cleaning Validation Method for Synthetic Peptide Drug Substances

---

Bhaskar K, Tirumal Datar, Taposh Gorella, Dr. Padmakar Wagh

Waters Corporation



---

## Abstract

This application note highlights the development of a simple, simultaneous, sensitive, and robust LC-MS/MS quantification of the therapeutic peptides.

### Benefits

- High sensitivity and accuracy achieved using chromatographic separation and the Xevo TQ-XS Mass Spectrometer, with quantitative performance from 1–1000 ng/mL for five peptides in single analytical run
- XBridge BEH Phenyl Column 2.5  $\mu$ m 2.1 x 100 mm for selectivity, high sensitivity, and narrow peak widths
- QuanRecovery LC-MS compatible sample vials with MaxPeak High Performance Surfaces mitigated non-specific binding, improved peptide recovery, while ensuring assay sensitivity, and reproducibility
- Simultaneous quantification of five diverse therapeutic peptides, using one analytical method, increasing laboratory efficiency

---

## Introduction

To ensure safety of a drug product, drug manufacturers must carefully control manufacturing processes, which includes monitoring active pharmaceutical ingredient (API), impurities and any other potential contaminants that are often a result of cross-contamination from previously manufactured process. To minimize potential cross-contamination, procedures for equipment cleaning after manufacturing processes are completely employed.

In fact, cleaning and cleaning validation are two activities that have the largest opportunity to prevent patient risk by ensuring that no cross-contamination can occur after every batch manufacturing. Cleaning validation is a documented evidence with a high degree of assurance that one can consistently clean a system or a piece of equipment to predetermined acceptable limits or established acceptable residue levels (ARLs).<sup>1,2</sup> Ineffective cleaning can lead to adulterated product which may be a result from previous product batches, cleaning agent or other extraneous material. Thus, the prime purpose of validating a cleaning process is to ensure the safety, efficacy or quality of drug product produced, while complying with current good manufacturing practices (CGMP) regulations, other federal and other standard regulations.<sup>3,4</sup>

During regulatory inspections, drug manufacturers focus a great deal of attention specifically directed to cleaning and cleaning validation of reaction chambers and multi-use purification systems. While ensuring utmost safety of drug product manufacturing is priority, minimizing downtime in manufacturing is also important in maintaining efficiency and avoiding negative economic impact due to idle equipment use. This puts increased pressure on rapidly developing analytical cleaning methods which are fast, sensitive, selective, and robust to detect the ARL of drug product or other suspected contaminants. While High Performance Liquid Chromatography (HPLC) coupled to Ultraviolet (UV) detection is the most common analytical tool for ARL determination, there is a growing need for analytical methodologies which can achieve more sensitive and selective detection.<sup>5-8</sup>

In fact, with the increase manufacturing of biological-based therapeutics, like peptides, with their high potency at lower dosage levels, the need for cleaning validations methods that can achieve very low ng/mL detection is often required. With their fast method development times and high specificity afforded by selective multiple reaction monitoring (MRM) fragments, liquid chromatography-mass spectrometry (LC-MS) ARL quantification for biological-based therapeutics are increasing. However, working with biotherapeutics presents increased analytical challenges compared to small molecules. due to their larger size, lower sensitivity, poor MS/MS fragmentation, and their propensity to suffer from significant adsorptive loss mainly from non-specific binding (NSB) and carryover issues. These challenges lead to long method development, less than ideal limits of quantification, and poor assay robustness. The work described here, uses a sample preparation strategy to maintain peptide solubility and use of LC-MS compatible sample storage vials with high performance surfaces to mitigate peptide loss due to NSB. In addition this, method highlights the use of a low dispersion UHPLC and a reversed-phase BEH Phenyl Column chemistry for chromatographic separation coupled to a sensitive tandem quadrupole mass spectrometer for simultaneous, sensitive, and accurate quantification of the five diverse peptides: semaglutide, liraglutide, glucagon, tetracosactide, and abaloparatide. The physiochemical properties, including amino acid sequence, molecular formula, molecular weight, isoelectric point (pI), and HPLC index for these peptides are listed in Table 1. This above-mentioned method achieves lower limits of quantification (LLOQs) of 1 ng/mL with a 5  $\mu$ L injection of prepared sample.

Name	Amino acid sequence	Molecular formula	Molecular weight	pI	HLB index
Semaglutide	DLSWIYLPUIQAV-CCUURXOWSA-N	C <sub>187</sub> H <sub>291</sub> N <sub>45</sub> O <sub>59</sub>	4113.641 g/mol	5.74	89.7
Liraglutide	HAEGTFTSDVSSYLEGQAAKEEFIAWLVRGRG	C <sub>172</sub> H <sub>265</sub> N <sub>43</sub> O <sub>51</sub>	3751.202 g/mol	5.74	97.0
Glucagon	HSQGTFTSDYSKYLDSSRAQDFVQWLMNT	C <sub>153</sub> H <sub>225</sub> N <sub>43</sub> O <sub>49</sub> S	3482.623 g/mol	7.34	86.0
Tetracosactide/cosyntropin	YSMEHFRWGKPVGKKRRPVKVYP	C <sub>136</sub> H <sub>210</sub> N <sub>40</sub> O <sub>31</sub> S	2932.587 g/mol	10.89	39.9
Abaloparatide	VSEHQLLHDKGKSIQDLRRRELLEKLLXKLHTA	C <sub>174</sub> H <sub>299</sub> N <sub>56</sub> O <sub>49</sub>	3959.649 g/mol	9.92	123.1

*Table 1. Physiochemical properties for the peptides: semaglutide, liraglutide, glucagon, tetracosactide, and abaloparatide.*

## Experimental

### Preparation Calibration Standards

An individual stock solution of 1000 µg/mL of the five peptides (Table 1) were prepared in 100% methanol. An intermediate mixed stock solution (10 µg/mL) was prepared by mixing equal aliquots of the 1000 µg/mL individual peptide solutions and dilution using a 50:50 water:acetonitrile solution containing 2% of formic acid. Sample from 1–1000 ng/mL were prepared by serial dilution with same diluent described above. All calibration curve standards, and blank (non-spiked) samples were prepared in duplicate. MS-grade difluoro acetic acid (DFA) and formic acid (FA) were used as mobile phase additives.

## Method Conditions

Parameters	Description
LC system	ACQUITY UPLC I-Class
Column	XBridge BEH Phenyl Column 2.5 µm, 2.1 × 100 mm
Column temperature	70 °C
Flow rate	0.2 mL/min
Injection volume	5 µL
Mobile phase	A: 0.4% Formic acid in water B: 0.1% Difluoro acetic acid in acetonitrile
LC wash solvent	Needle wash and purge: acetonitrile: ME OH: IPA: water (1:1:1:1) with 0.1% trifluoro ethanol and 0.4% formic acid  Seal wash: 95:5 V/V water: methanol

## LC Gradient

Step	Flow rate	Time	%A	%B
Initial	0.2	0.00	60	40
1	0.2	6.00	10	90
2	0.8	6.50	10	90
3	0.8	7.00	5	95
4	0.8	8.00	5	95
5	0.8	12.0	50	50
6	0.8	14.0	20	80
7	0.8	14.4	60	40
8	0.2	14.5	60	40
9	0.2	15.0	60	40

## MS Conditions

MS system	Xevo TQ-XS
Source	ESI+
Capillary voltage	3.0 kV
Source temp.	150 °C
Desolvation temp.	600 °C
Desolvation gas flow	1000 L/Hr
Cone gas flow	100 L/Hr
Nebulizer	3.5 Bar

Analyte	Precursor	Fragment	Cone (V)	Collision (eV)
Semaglutide	1029.35	135.93	20	30
Liraglutide	938.77	110.02	50	30
Glucagon	697.25	694.06	54	12
Tetracosactide	489.83	537.262	40	10
Abaloparatide	566.81	632.28	50	10

---

Table 3. Peptide MRM transitions, cone voltages, and collision energies used for quantitation.

## Data Management

LC-MS software: MassLynx v4.2

Quantification software: TargetLynx

---

## Results and Discussion

All steps in the sample preparation, LC, and MS method were optimized during method development to overcome issues of poor solubility, NSB, peak tailing, and carryover.

## Mass Spectrometry

LC-MS/MS quantification was performed using the Xevo TQ-XS Tandem Quadrupole MS (ESI+). During method development, several multiply charged precursors were observed for the various peptides. The most intense precursor charge state for each peptide was chosen for MRM analysis. This corresponded to the 4+ for semaglutide, 4+ for liraglutide, 5+ for glucagon, 6+ for tetracosactide, and the 7+ for abaloparatide. Additionally, use of highly specific b/y ion fragments yielded significantly improved specificity. Optimized MS conditions are listed in the experimental section, with MRM transition settings listed in Table 3.

## Liquid Chromatography

Chromatographic separation was achieved by using an XBridge BEH Phenyl 2.5  $\mu\text{m}$ , 2.1 x 100 mm Column. Water containing 0.4 % FA and acetonitrile containing 0.1% DFA were used as mobile phases. For analysis, a 6-minute gradient was employed. To mitigate on-column peptide carryover of the most problematic peptide, liraglutide, the flow rate was increased while alternating mobile phase composition following peptide separation. Use of the reversed-phase phenyl column and DFA mobile phase improved assay sensitivity, provided excellent peak shape, and chromatographic resolution for the diversified peptides in this assay. This chromatographic performance of the LLOQ (1 ng/mL) as compared to blank sample is demonstrated in Figure 1. For all peptides, signal-to-noise (S/N) was  $\geq 10$  at the LLOQ.



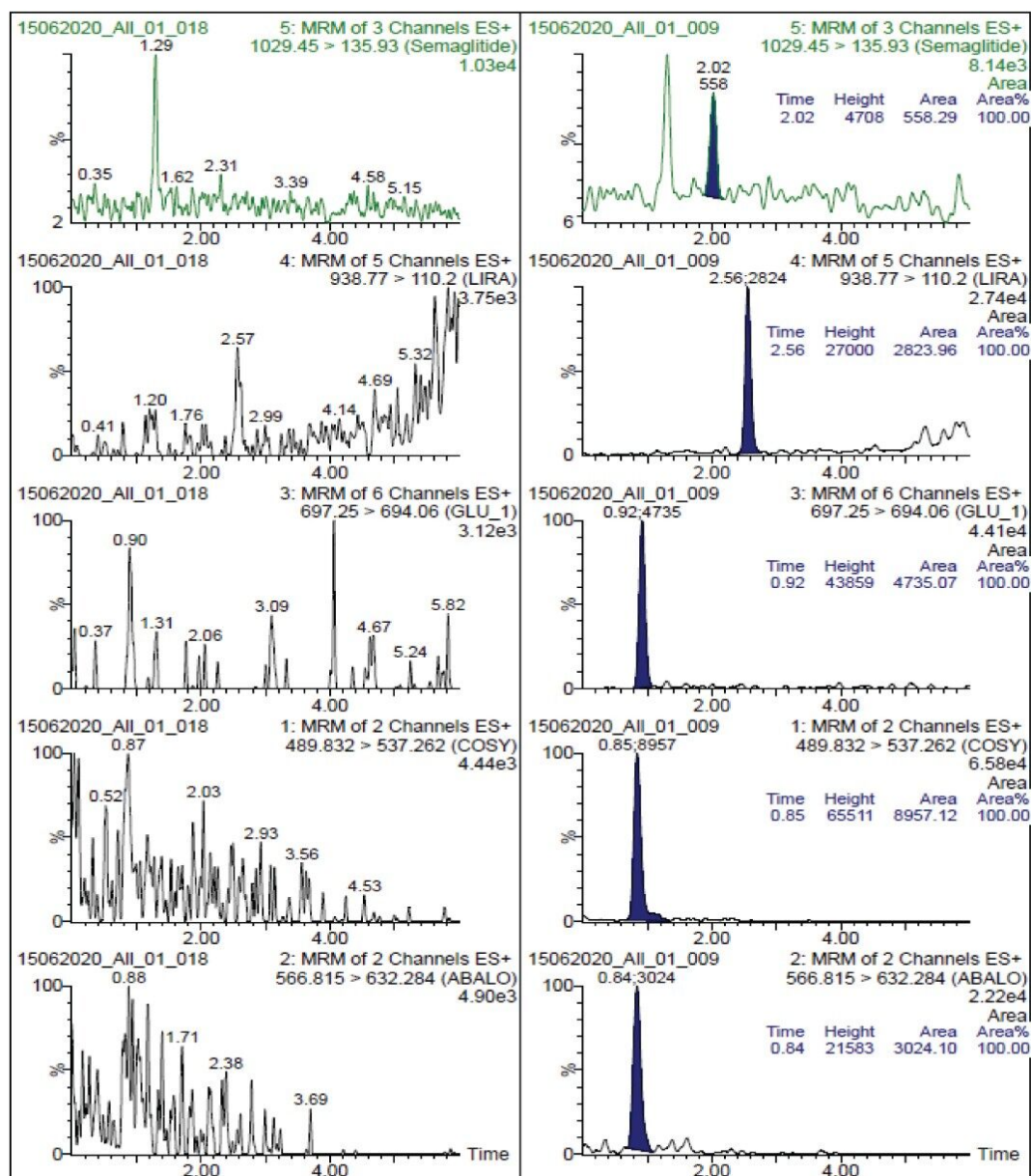


Figure 1. Representative LLOQ and Blk chromatograms to check carryover for the peptides: semaglutide, liraglutide, glucagon, tetracosactide, and abaloparatide.

## Calibration Curve, Linearity, Precision, and Accuracy

Using only 5  $\mu\text{L}$  of sample and the described LC-MS method, quantification limits of 1 ng/mL of the peptides was achieved. Dynamic ranges of the calibration curves were 1–1000 ng/mL with  $R^2$  values  $>0.99$  (1/x<sup>2</sup> weighted regression). Accuracies of all calibration points were between 88–112%, meeting recommended performance of 85–115%. This performance is highlighted in Table 4 and illustrated in Figure 2.



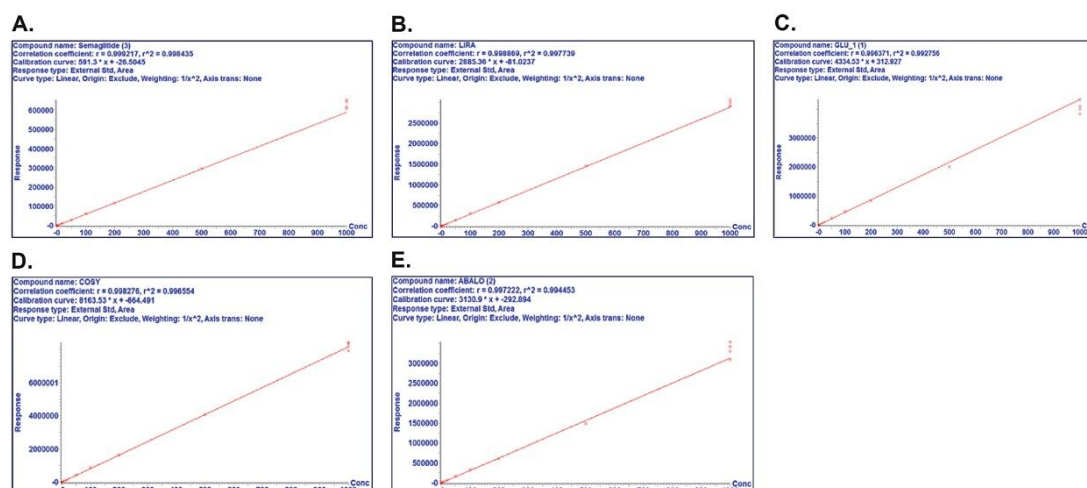


Figure 2. Representative calibration curves highlighting dynamic ranges from 1 ng/mL to 1000 ng/mL for the peptides semaglutide (A), liraglutide (B), glucagon (C), tetracosactide/cosyntropin (D), and abaloparatide (E).

Analyte	Standard curve range	Calibration curve weighting	Calibration curve linearity ( $R^2$ )	Calibration curve accuracy (%)	LOQ (1 ng/mL) signal/noise
Semaglutide	1 ng/mL to 1,000 ng/mL	1/X2	0.998	88 to 107%	23
Liraglutide			0.996	96 to 108%	85
Glucagon			0.984	88 to 112%	102
Tetracosactide/Cosyntropin			0.996	94 to 109%	70
Abaloparatide			0.994	88 to 109%	41

Table 4. Representative quantification performance for semaglutide, liraglutide, glucagon, tetracosactide, and abaloparatide.

## Conclusion

This application note highlights the development of a simple, simultaneous, sensitive, and robust LC-MS/MS quantification of the therapeutic peptides: semaglutide, liraglutide, glucagon, tetracosactide, and abaloparatide. This method demonstrates its fit-for-purpose to support cleaning validation activities required during drug manufacturing. The developed method achieves LLOQs 1 ng/mL for all five peptides, with linear dynamic ranges from 1–1000 ng/mL. The high sensitivity and quantitative performance of the method was

attributed to careful sample preparation and LC-MS method optimization. Using carefully chosen sample diluents to maintain peptide solubility and use of QuanRecovery sample vials with MaxPeak technology to mitigate peptide loss due to NSB facilitated high recovery of all peptides. Use of a low dispersion and high resolution UPLC chromatographic separation with the ACQUITY UPLC I-Class System and XBridge BEH Phenyl Column, coupled to the Xevo TQ-XS Tandem Quadrupole Mass Spectrometer, ensured great peak shape, with narrow peak widths <10 seconds, easily achieving 1 ng/mL detection with standards. This developed method has demonstrated its fit-for-purpose use in support of drug research and manufacturing.

---

## References

1. <http://www.pharmtech.com/enhancing-drug-development-applying-lc-ms-ms-cleaning-validation-manufacturing-equipment>. <<http://www.pharmtech.com/enhancing-drug-development-applying-lc-ms-ms-cleaning-validation-manufacturing-equipment>>
2. Sharma A, Vaghela J.S, Sharma P, Sharma B, Agrawal M, Jagiwala S. Cleaning Validation in Pharmaceutical Industry. A Comprehensive Approach; pharmatutor-art-1755.
3. Robert Fredrick. The Basic Facts of Cleaning Validation. Canada: Pharmainfo.net; 2004. Available from <http://www.pharmainfo.net/reviews/basic-facts-cleaning-validation>.
4. LeBlanc DA. Establishing Scientifically Justified Acceptance Criteria for Cleaning Validation of Finished Drug Products. *Pharm Tech*. 1998; 22(10): 136–148.
5. Fourman GL, Mullen MV. Determining Cleaning Validation Acceptance Limits for Pharmaceutical Manufacturing Operations. *Pharm Tech*. 1993; 17: 54–60.
6. M.B. Boca, Z. Apostolides, E. Pretorius. A Validated HPLC Method for Determining Residues of a Dual Active Ingredient Anti-Malarial Drug on Manufacturing Equipment Surfaces. *J. Pharm. Biomed. Anal.* 37 (3), 461–468 (2005).
7. M.J. Nozal, *et al.* Development and Validation of an LC Assay for Sumatriptan Succinate Residues on Surfaces in the Manufacture of Pharmaceuticals. *J. Pharm. Biomed. Anal.* 30 (2), 285–291 (2002).
8. J. Lambropoulos, G.A. Spanos, N.V. Lazaridis. Development and Validation of an HPLC Assay for Fentanyl, Alfentanil, and Sufentanil in Swab Samples. *J. Pharm. Biomed. Anal.* 23 (2-3), 421–428 (2000).

9. T. Mirza, *et al.* Cleaning Level Acceptance Criteria and a HighPressure Liquid Chromatography Procedure for the Assay of Meclizine Hydrochloride Residue in Swabs Collected from Pharmaceutical Manufacturing Equipment Surfaces. *J. Pharm. Biomed Anal.* 19 (5), 747–756 (1999).
  10. D.A. LeBlanc. Establishing Scientifically Justified Acceptance Criteria for Cleaning Validation of APIs. *Pharm Technol.* 24 (10), 160–168 (2000).
  11. D.A. LeBlanc. Establishing Scientifically Justified Acceptance Criteria for Cleaning Validation of Finished Drug Products. *Pharm Technol.* 22 (10), 136–148 (1998).
- 

## Featured Products

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

[Xevo TQ-XS Triple Quadrupole Mass Spectrometry <https://www.waters.com/134889751>](https://www.waters.com/134889751)

[MassLynx MS Software <https://www.waters.com/513662>](https://www.waters.com/513662)

[TargetLynx <https://www.waters.com/513791>](https://www.waters.com/513791)

720007035, October 2020