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アプリケーションノート

# Application of LC-UV/MS Workflows to Increase Efficiency in Impurity Profiling of GLP-1 Analogs

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*Published on May 16, 2025*

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## Abstract

Glucagon-like-peptide-1 (GLP-1) receptor agonists have gained significant attention recently as a metabolic regulator for treating type 2 diabetes and obesity. The increased visibility as a weight-loss treatment has increased demand and propelled research into new analogs and delivery methods. The surge in demand has introduced analytical challenges from peptide synthesis, impurity profiling, and stability monitoring, as manufacturers look to drive efficiency and scale-up production. LC-UV/MS platforms offer the potential to alleviate the burden on analytical laboratories during late-stage method development and early manufacturing phases of GLP-1 analogs. Access to orthogonal mass information allows support labs to make informed decisions more efficiently to reduce errors and increase overall productivity.

In this study, the unique capabilities of a LC-UV/MS workflow to accelerate analysis are demonstrated in the identification of impurities related to GLP-1 analogs that underwent chemical and thermal stress conditions.

## Benefits

- Orthogonal mass information provides data-driven insights on forced degradation impurities generated via oxidation, pH, and thermal stress.
- The ACQUITY™ QDa™ II Mass Detector enhances the capabilities of analytical labs in raw material screening, process control, lot release, and stability monitoring.
- LC-UV/MS workflow expedites risk-based decision-making and increases overall productivity in the lab.

# Introduction

Glucagon-like-peptide-1 receptor agonists (GLP-1 RAs) have been widely used as medications to treat type 2 diabetes and obesity. While relatively simple in structure as a short chain of amino acids, the production and modification of GLP-1 analogs can be quite varied (Figure 1A). Briefly, the peptide backbone can be produced via recombinant DNA techniques or chemical synthesis (in solid phase, liquid phase, or a hybrid of both). Modifications, if present, are made via chemical conjugation to add a fatty acid chain or protein fragment to prolong half-life or achieve other desired pharmacokinetic and/or pharmacodynamic effects.[1]

As GLP-1 analogs become increasingly complex, particularly with conjugates, new challenges arise in identifying and defining acceptance criteria in their production. To meet product demand while maintaining regulatory expectations for safety and quality, manufacturers need reliable and easy methods that can be logistically deployed to support the production of GLP-1 analogs.

LC-UV/MS platforms offer the potential to alleviate the workload on analytical laboratories during method development and manufacturing activity while maintaining drug quality and safety. Access to orthogonal mass information allows support labs to make informed decisions more efficiently during method development and manufacturing to reduce errors and increase overall productivity. This study aims to demonstrate the inherent value of mass information in workflows related to raw material screening, process control, lot release, and stability monitoring to expedite decision-making and increase overall lab productivity. This will be accomplished through a data-driven discussion with use cases presented for GLP-1 analogs that undergo forced degradation conditions including oxidation, pH, and thermal stress.

# Experimental

Research grade GLP-1 analogs exenatide and tirzepatide were purchased from multiple vendors. Exenatide stock was prepared at 0.25 mg/mL using acetate buffer at pH 4.5. Tirzepatide stock was prepared at 5 mg/mL using sodium phosphate and sodium chloride buffer at pH 7.4. The stock concentration and buffer were chosen to match the formulation of corresponding drug products with minor adjustment. Oxidation of peptides was completed using hydrogen peroxide. Exenatide was digested with RapiZyme™ Trypsin at 37 °C for 30 minutes (peptide: enzyme weight ratio 20:1). Trypsin was inactivated by adding 0.1% formic acid/water after the digestion. Thermal degradation occurred at 50 °C. Samples were analyzed at 0.1 mg/mL with the ACQUITY QDa II Mass Detector.[3]

## ACQUITY QDa II Mass Detector Analysis

| | |
|---|---|
| LC system: | Arc™ Premier System (QSM) |
| Detection: | TUV, λ = 220 nm<br>ACQUITY QDa II Mass Detector |
| Column: | XSelect™ Premier Peptide CSH™ $C_{18}$ Column, 130Å, 2.5 µm, 4.6 x 100 mm (p/n: 186009908 <https://www.waters.com/nextgen/global/shop/columns/186009908-xselect-premier-peptide-csh-c18-column-130a-25--m--46-x-100-mm-1.html> ) (+eConnect™ 186009908RF <https://www.waters.com/nextgen/global/shop/columns/186009908rf-xselect-premier-peptide-csh-c18-column-130-a-25--m--46-x-100-mm-.html> ) |
| Column temperature: | 60 °C |
| Sample temperature: | 10 °C |
| Injection volume: | 10 µL |
| Flow rate: | 0.96 mL/min |

| | |
|---|---|
| Mobile phase: | A: 0.1% formic acid in water<br>B: 0.1% formic acid in acetonitrile |
| Chromatography software: | Empower™ Chromatography Data System (CDS) 3.8.1 |

## ACQUITY QDa II Mass Detector Settings

| | |
|---|---|
| Ionization mode: | ESI+ |
| Acquisition mode: | Full scan |
| Acquisition range: | 250–1500 *m/z* |
| Scan rate: | 5 Hz |
| Capillary voltage: | 1.5 kV |
| Cone voltage: | 15 V |
| Probe temperature: | 600 °C |

## LC Gradient Table

| Time (min) | Flow (mL/min) | %A | %B | %C | %D | Curve |
|---|---|---|---|---|---|---|
| initial | 0.96 | 99.0 | 1.0 | 0 | 0 | initial |
| 2.00 | 0.96 | 99.0 | 1.0 | 0 | 0 | 6 |
| 22.00 | 0.96 | 45.0 | 55.0 | 0 | 0 | 6 |
| 25.00 | 0.96 | 5.0 | 95.0 | 0 | 0 | 6 |
| 26.00 | 0.96 | 5.0 | 95.0 | 0 | 0 | 6 |
| 28.00 | 0.96 | 99.0 | 1.0 | 0 | 0 | 6 |
| 40.00 | 0.96 | 99.0 | 1.0 | 0 | 0 | 6 |

## Results and Discussion

### Manufacturing Environments

Analytical labs supporting manufacturing processes are primarily driven by UV-based detectors that offer a cost-effective solution with broad sample compatibility and a low barrier of adoption due to their linear response and ease of use. In contrast, MS-based solutions are often viewed as overly complex, requiring frequent maintenance and user expertise to operate despite the potential benefit they could bring to manufacturing environments. The ACQUITY QDa II Mass Detector (Figure 1B), is engineered as an affordable compact mass detector that can be configured in-line with UV-based workflows to provide orthogonal mass data with the simplicity of on/off operation.[2] Integration with the Empower Chromatography Data System as a compliant-ready, scalable software solution for instrument control, data acquisition, review, and reporting with full audit trail capabilities makes it well suited for manufacturing environments. To demonstrate the intrinsic value of orthogonal mass data, GLP-1 analogs were chemically and thermally stressed as an extension of impurity profiling frequently carried out in stability testing and comparability studies.

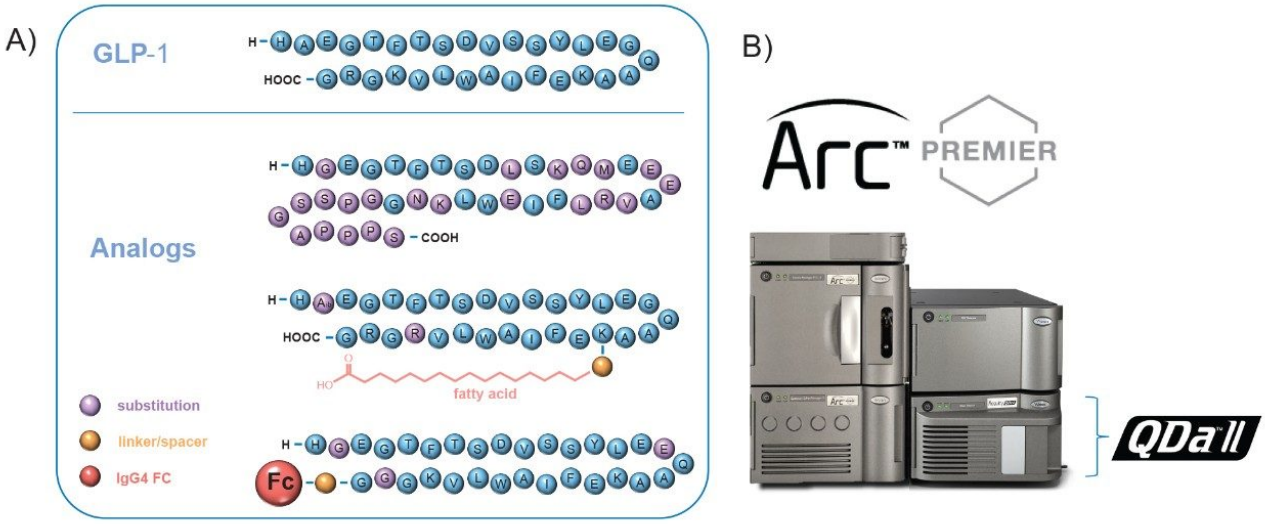


*Figure 1. A) Illustrative example of structure for various GLP-1 analogs types. B) The ACQUITY QDa II Mass Detector configured as an in-line detector with the ACQUITY Premier UPLC System.*

### Stability Testing

As part of development and manufacturing, drug substances undergo forced degradation using thermal,

chemical, and UV-stress to determine stability, shelf-life, and storage conditions. In this setting, LC-UV/MS workflows can be utilized to quickly identify degradants and potential stability indicating attributes for routine monitoring. As an example, exenatide was placed under thermal stress for a 30-day period and analyzed using an LC-UV/MS workflow. As shown in Figure 2A, several new peaks were observed in the UV chromatogram of the thermally stressed sample with 7 peaks exhibiting a significantly elevated peak area. As shown in the bottom panel, the ACQUITY QDa II Mass Detector is readily capable of detecting all observed peaks enabling the use of orthogonal mass data to putatively identify degradants. An example is shown for peak 2 in which a related ion series ($[M+3H]^{+3}$ and $[M+4H]^{+4}$) was observed in the spectral data and determined to correlate to a mass of 3,254.1 Da. Using this information, a quick examination of the amino acid sequence indicated this mass potentially represents a single cleavage occurring between the aspartic acid and leucine residues of exenatide (Figure 2B). Extracted ion chromatograms (XICs) were performed on full spectrum data of the thermally stressed sample. As shown in Figure 2B, a matching set of peaks were identified for the $[M+4H]^{+4}$ and $[M+2H]^{+2}$ charge states related to the peptide fragments, potentially indicating a site of thermal instability that may need further development in terms of sequence and activity.

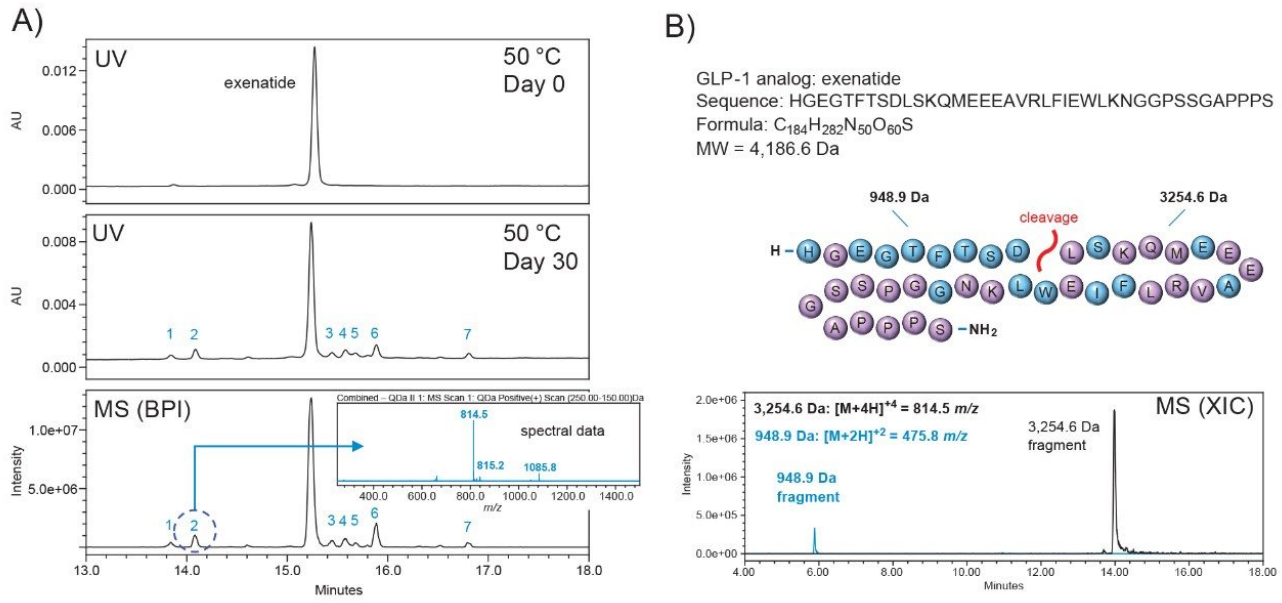


*Figure 2. A) An Exenatide control sample (top panel) was exposed to thermal stress for a 30-day period (middle panel) with 7 "new" peaks observed in the stressed sample. Spectral data was used to calculate the observed mass of each peak for putative identity of degradants (bottom panel). B) As an example, peak 2 was determined to have an observed mass of 3,254.1 Da. which correlates to a non-enzymatic cleavage occurring between aspartic acid and leucine residues based on GLP-1 sequence information and was confirmed with retention time and mass using extracted ion chromatograms.*

## Orthogonal Confirmation

While this example demonstrates how orthogonal mass data can be utilized to expedite the analysis of GLP-1 analogs, another benefit of LC-UV/MS workflows is the ability to cross-correlate observations in analyses. An example of this is shown in Figure 3. Exenatide sample was placed under oxidative stress using a dilute solution of hydrogen peroxide. As shown in Figure 3A, the stressed sample indicates exenatide is predominantly oxidized with a +16 Da difference observed in the spectral data. The spectral signature of the ion series and retention time shift correlate to peak 1 of the thermally stressed data (Figure 3, bottom panel) indicating the drug substance may undergo a degree of oxidation when exposed to elevated temperatures. While this data is valuable to corroborate observation, LC-UV/MS workflows offer the capability to support more informative analyses as part of method development and manufacturing activities.

To demonstrate this, both samples were analyzed at a peptide level to gain more insights into the potential site

of oxidation. As shown in Figure 3B, using RapiZyme Trypsin, a quick 30-minute enzymatic treatment was performed to generate four unique peptide fragments, two of which contain methionine and tryptophan residues (circled red), which are known to be susceptible to oxidation. As shown in the chromatograms, four unique peaks are observed for the RapiZyme Trypsin treated samples when separated using a reversed-phase technique. Peptide fragments in the control sample were quickly identified based on the mass data, an example of which is shown for peak 2 (inset) with the $[M+H]^+$ ion agreeing well with the expected mass for the T2 fragment. When compared to the stressed sample, all peaks agreed in retention time and mass data except for peak 2. Closer inspection of the mass data related to the "new" peak at 4.5 minutes in the stressed sample in conjunction with the absence of peak 2 when compared to the control, indicates this peak is likely related to the oxidized form of the fragment T2. This observation suggests that the methionine in the fragment T2 is more sensitive to oxidative stress, providing deeper insights into drug stability.

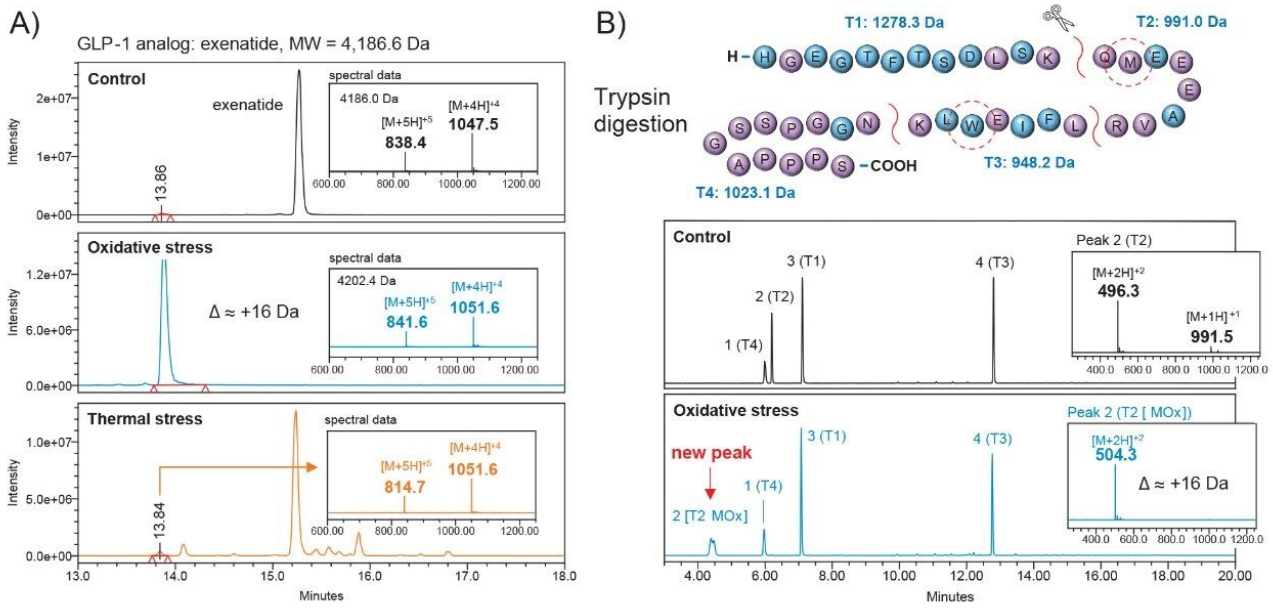


*Figure 3. A) Exenatide control (top panel) was subjected to oxidative stress using hydrogen peroxide. Stressed sample showed a retention time shift (-1.3 min) and mass difference (+16Da) (middle panel). This data was cross-correlated to confirm oxidation degradants in the thermally stressed sample (bottom panel). B) Control and oxidatively stressed samples of exenatide were enzymatically treated. The new peak in the stress sample was putatively identified as the oxidized form of the T2 peptide fragment based on mass data.*

## Specificity

LC-UV/MS-enabled workflows offer a unique advantage of specificity when compared to UV-based workflows alone. This can prove useful when investigating stability indicating impurities that may exhibit unique properties as in the case of fatty acid conjugated GLP-1 analogs. As shown in Figure 4, fatty acids increase the complexity of profiling experiments through the introduction of non-native linkers and potentially more hydrophobic conjugates. This is demonstrated in the UV chromatograms of tirzepatide, which exhibits a longer retention time with elution occurring at organic compositions above 50%. This can prove to be challenging when investigating the stability of the fatty acid conjugate and linker species. To demonstrate this, a sample of tirzepatide was thermally stressed. As shown in Figure 4B, multiple low-abundant peaks were observed in the UV chromatogram of the stressed sample. Identification of the fatty acid using UV data alone would be challenging in the absence of a synthetic standard to compare against. However, as shown in the bottom panel of stressed data, MS data can be used to confirm species of interest such as the fatty acid conjugate, which was observed at 26 minutes (342.5 *m/z*) along with the remaining peptide fragment (1123.5 *m/z*) at 17 minutes. Interestingly, based on the UV chromatogram, a minor peak eluting at 26 minutes under 95% organic conditions, likely corresponding to the fatty acid, might have been dismissed as a reconditioning artifact if MS data had not been available. In this case, the specificity provided by the ACQUITY QDa II Mass Detector allowed for rapid identification of the conjugate species by mass, confirming a potential degradation pathway of the drug substance.

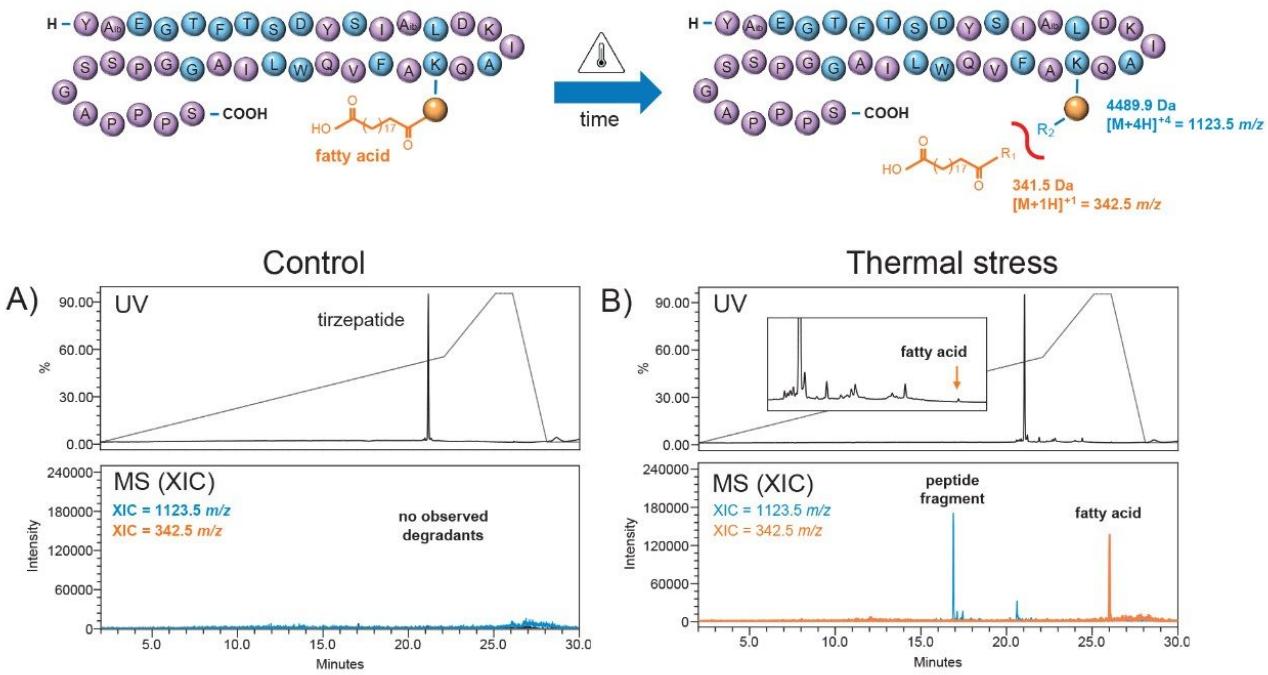


*Figure 4. A) Control and B) thermally stressed tirzepatide were compared to investigate the linker and conjugate stability under thermal stress. Non-enzymatic cleavage fragments of the fatty acid and peptide fragment were confirmed by mass using extracted ion chromatograms.*

## Conclusion

In this study, it was demonstrated how the ACQUITY QDa II Mass Detector can be effectively utilized to support impurity profiling and stability testing activity associated in the production of GLP-1 analogs with the following observations:

- Compact mass detectors such as the ACQUITY QDa II Mass Detector that feature on/off simplicity and can be easily integrated into existing LC-UV platforms have the potential to lower adoption barriers associated with MS and increase the analytical capabilities of supporting labs.
- Access to mass data provides deeper understanding of drug product behaviors enabling more insightful decision-making during the development and manufacturing process.
- In-line acquisition of complementary orthogonal mass data increases confidence in results and product

knowledge for improved product safety and regulatory compliance while maintaining productivity.

## References


1. Yu, M. et al. Battle of GLP-1 Delivery Technologies. *Advanced drug delivery reviews*. (2018) 130:113-130. DOI: 10.1016/j.addr.2018.07.009.
2. Birdsall. R.E., Du, X., Nyholm, K. Extending the Analytics of Biopharmaceutical QA/QC Labs with the ACQUITY™ QDa™ II Mass Detector. Waters Application Note. 720008386. June 2024.
3. Han, D., Ippoliti, S., Birdsall. R.E., Nyholm, K. Accelerating Method Development and Manufacturing of GLP-1 Analogs with LC-UV/MS. Waters Application Note. 720008800. May 2025.


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720008812, May 2025

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