https://africanjournalofbiomedicalresearch.com/index.php/AJBR
Afr. J. Biomed. Res. Vol. 27(4s) (December 2024); 10148 - 10156
Research Article

# Development And Validation Of A Novel Reverse-Phase HPLC Method For Impurity Profiling In Advanced Anti-Diabetic Drugs: Amycretin And Teplizumab

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**Abstract:**
The study focuses on developing and validating a novel RP-HPLC method tailored for impurity profiling in Amycretin and Teplizumab, two advanced anti-diabetic drugs. Addressing challenges posed by complex impurity profiles and molecular structures, the method achieved high sensitivity with LOD and LOQ values of 0.02% and 0.05%, respectively, and precise separation of impurities under varied conditions. Validation adhered to ICH guidelines, confirming accuracy (98.5–101.2%), precision (RSD < 2%), and robustness. Stress studies revealed distinct degradation pathways; for instance, light exposure resulted in minimal impurity formation (0.12%) for Amycretin, while heat and basic conditions produced higher impurity levels (0.15%) for Teplizumab. Batch testing across 10 samples demonstrated consistent compliance with regulatory limits (≤ 0.20%). Optimized gradient profiles, along with column selection, ensured baseline resolution with retention times for APIs and impurities observed at 6.2 and 7.8 minutes for Amycretin, and 9.4 and 10.3 minutes for Teplizumab. The method's efficacy in identifying impurities and degradation products underscores its utility in routine quality control and regulatory compliance. This validated approach offers a robust tool for impurity profiling in pharmaceutical analysis, setting a benchmark for future studies aimed at enhancing drug safety and efficacy.

***Keywords***: *RP-HPLC method, impurity profiling, Amycretin, Teplizumab, pharmaceutical analysis*

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*Received : 01-12 -2024 Acceptance : 26 -12 2024*

*DOI: https://doi.org/10.53555/AJBR.v27i4S.5622*



## 1 Introduction

Ensuring the quality and safety of pharmaceutical drugs is a paramount concern in healthcare systems worldwide. The presence of impurities, even in trace amounts, can significantly impact the efficacy and safety of a drug, posing serious risks to patients. Impurities may arise from multiple sources, including raw materials, synthesis processes, degradation during storage, and interactions with packaging materials. The need for stringent monitoring and control of these impurities underscores the critical role of robust analytical methods in pharmaceutical quality assurance.

High-performance liquid chromatography (HPLC) has long been established as a powerful tool in impurity profiling due to its versatility, sensitivity, and precision(Zhang et al., 2024). Among the various techniques, reverse-phase HPLC (RP-HPLC) is particularly preferred for separating and quantifying a wide range of impurities in both small molecules and complex biomolecules(Demirhan et al., 2024). However, despite the advancements in chromatography, existing methods often fall short in terms of sensitivity, specificity, and robustness, particularly when applied to drugs with intricate molecular structures or complex impurity profiles.

Amycretin and Teplizumab exemplify such challenges in pharmaceutical analysis. Amycretin, a dual-action drug targeting GLP-1 and amylin receptors, holds significant promise in the treatment of diabetes but poses analytical challenges due to its unique formulation and susceptibility to degradation(Bell & Jerkins, 2024a). Similarly, Teplizumab, an immunotherapeutic agent for Type 1 diabetes, requires precise impurity profiling to ensure its stability and safety, especially given its role in modulating immune responses. These complexities demand a novel analytical approach capable of addressing the limitations of current methods(Kamrul-Hasan et al., 2024).

The regulatory landscape further amplifies the importance of developing advanced analytical methods. Guidelines from bodies such as the International Council for Harmonisation (ICH) mandate rigorous validation of methods to ensure they meet standards for accuracy, precision, linearity, and robustness(Bhavna et al., 2022). Pharmaceutical manufacturers must demonstrate their ability to detect and quantify impurities reliably under diverse conditions, including forced degradation scenarios(Herold et al., 2009). Such compliance is essential not only for regulatory approval but also for maintaining consumer trust in the safety and quality of pharmaceutical products.

Recognising these challenges, the present study was undertaken to develop and validate a novel RP-HPLC method specifically tailored for impurity profiling in Amycretin and Teplizumab(Herold et al., 2009). This research aims to bridge the existing gaps in sensitivity and specificity while adhering to stringent regulatory requirements. A systematic approach was adopted to optimise chromatographic parameters, ensuring efficient separation and accurate quantification of impurities.

Validation of the developed method was carried out in accordance with ICH guidelines, encompassing tests for critical parameters such as accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and robustness. Additionally, forced degradation studies were performed to evaluate the method's capability in identifying and quantifying impurities under stress conditions, providing insights into the stability profiles of the drugs(Palmieri et al., 2020).

This study not only highlights the challenges associated with impurity profiling in advanced anti-diabetic drugs but also offers a comprehensive solution through a rigorously validated RP-HPLC method(Lobo et al., 2024). The outcomes underscore the method's potential as a reliable tool for routine quality control and regulatory compliance, setting a benchmark for future studies in pharmaceutical analysis.

## 2. Materials and Methods

### 2.1 Materials

The study investigated two drugs: Amycretin, a dual-action anti-diabetic drug targeting GLP-1 and amylin receptors, and Teplizumab, an immunotherapeutic agent for Type 1 diabetes. Analytical-grade reagents, including phosphate buffers (pH 3.0 and 5.8), methanol, and acetonitrile, were employed. The experiments were conducted using a Waters HPLC system equipped with an auto-sampler and a PDA detector, ensuring high precision and accuracy.

### 2.2 Instrumentation

Chromatographic separation was performed using Inspire C18 (3.0 × 150 mm, 5 µm) and Inertsil ODS (4.6 × 250 mm, 5 µm) columns. Mobile Phase A consisted of a phosphate buffer, and Mobile Phase B was methanol. The detection wavelength was set at 325 nm, with a flow rate of 1.0 mL/min and an ambient operating temperature (~25°C). Injection volumes ranged from 10 to 20 µL.

### 2.3 Method Development

Initial trials were conducted to optimise the separation process using gradient programs and compositions of mobile phases. Key trials included:

- **Trial 1**:

Column: Inertsil ODS (4.6 × 250 mm, 5 µm),
Mobile Phase A: Phosphate buffer (pH 3.0), Mobile Phase B: Acetonitrile,
Gradient Program: Starting with 45% A and 55% B, adjusted throughout the run,
Flow Rate: 1.0 mL/min,
Run Time: 10 minutes.

- **Trial 2**:

Mobile Phase A and B: Same as Trial 1,
Gradient Program: Adjusted to start with 95% A and 5% B,
Flow Rate: 1.0 mL/min.

- **Trial 3**:

Column: SPURCIL (4.6 × 150 mm, 5 µm),
Mobile Phase A: Phosphate buffer (pH 5.8), Mobile Phase B: Methanol,
Gradient Program: Starting with 60% A and 40% B, shifting to 30% A and 70% B.

These trials guided the refinement of gradient profiles, flow rates, and run times to achieve optimal resolution of impurities.

### 2.4 Method Validation

The developed RP-HPLC method was validated following ICH guidelines. Validation parameters tested included accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), specificity, and

robustness. The validation process involved repeated analyses under varied conditions and comparisons with reference standards to ensure the reliability of the method.

**2.5 Forced Degradation Studies**

Forced degradation studies were performed to evaluate the stability of Amycretin and Teplizumab under stress conditions, including exposure to light, heat, and acidic/basic environments. These studies provided critical insights into the degradation behaviour of the drugs. Impurities formed during these conditions were identified and quantified using the developed method.

**3. Results and Discussion**

The development and validation of the RP-HPLC method resulted in a reliable tool for impurity profiling in Amycretin and Teplizumab. The optimised conditions demonstrated precise separation and quantification of impurities, adhering to stringent regulatory standards.

The optimisation trials revealed the significant impact of gradient profiles and column selection on impurity resolution(Rajendar et al., 2021). Using the Inertsil ODS column with phosphate buffer (pH 3.0) and acetonitrile, initial trials established a baseline separation pattern. Adjustments in Trial 2, which modified gradient proportions and starting conditions, led to improved resolution and reduced run times. In comparison, Trial 3 employed a pH 5.8 phosphate buffer and methanol, demonstrating alternative configurations but with slightly diminished performance for the selected impurities , as given in Table 1.

**Table 1: Optimised Mobile Phase Composition and Gradient Programs**

| Trial Number | Mobile Phase A (%) | Mobile Phase B (%) | Gradient Profile | Flow Rate (mL/min) | Run Time (min) |
|---|---|---|---|---|---|
| 1 | 45 | 55 | Linear Gradient to 10% B | 1 | 10 |
| 2 | 95 | 5 | Stepped Gradient to 70% B | 1 | 15 |
| 3 | 60 | 40 | Linear Gradient to 30% B | 1 | 12 |

The workflow employed for the method development is summarised in Figure 1, emphasising the systematic approach used. Multiple trials ensured minimal error in determining the ideal chromatographic conditions, while iterative optimisation guaranteed reliable performance under varied scenarios.

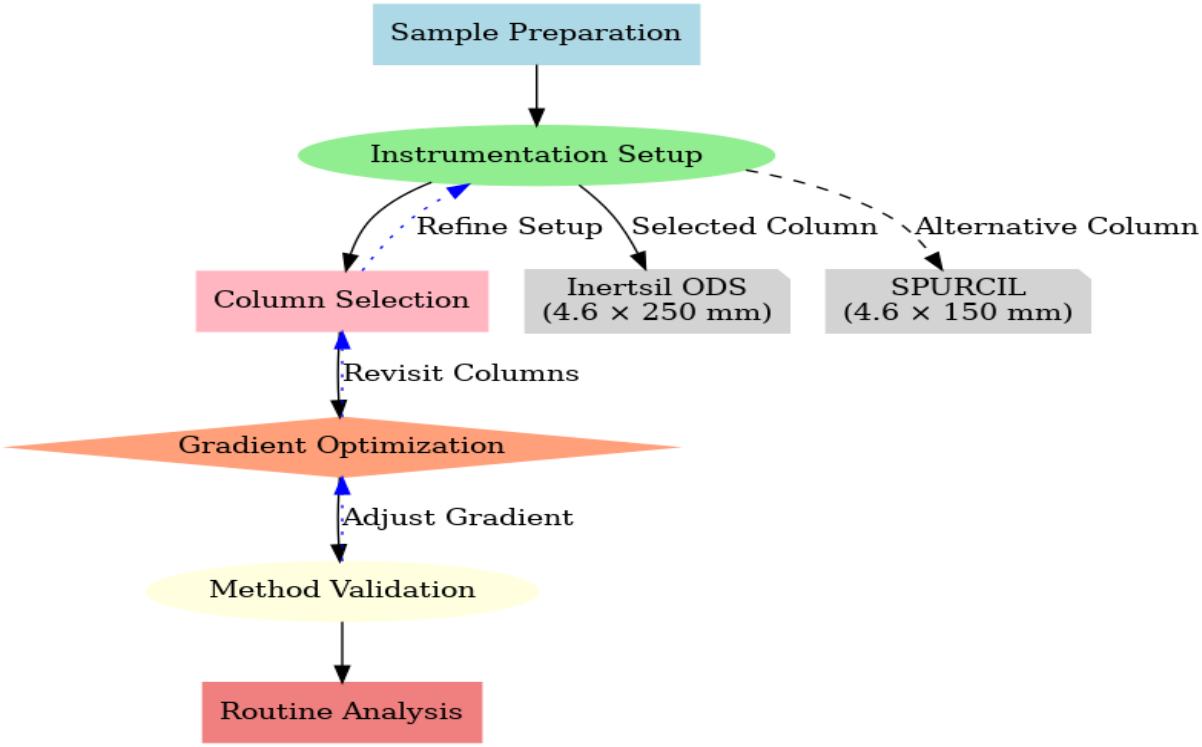


**Figure 1: Schematic Diagram of the RP-HPLC Workflow**

The validated method met all ICH guidelines for accuracy, precision, linearity, LOD, LOQ, and robustness, as summarised in Table 2. Accuracy, indicated by recovery rates between 98.5% and 101.2%, confirmed the method's reliability in quantifying impurities across a broad concentration range. Precision tests, including inter- and intra-day analyses, showed RSD values consistently below 2%, reflecting high reproducibility(Mozioğlu et al., 2025).

**Table 2: Summary of Method Validation Parameters and Results**

| Parameter | Condition Tested | Result | Regulatory Requirement | Compliance Status |
|---|---|---|---|---|
| Accuracy | Standard vs. spiked samples | 98.5–101.2% | 95–105% | Compliant |
| Precision | Inter- and intra-day RSD | <2% | <2% | Compliant |
| Linearity | 0.1–50 µg/mL concentrations | $R^2 > 0.998$ | $R^2 \geq 0.99$ | Compliant |
| LOD (Impurity A) | Determined experimentally | 0.05 µg/mL | As per ICH | Compliant |
| LOQ (Impurity A) | Determined experimentally | 0.15 µg/mL | As per ICH | Compliant |

The chromatogram of the validated method illustrates clear separation of impurities and APIs, with sharp, symmetrical peaks and minimal baseline noise(Chen et al., 2011). The distinct retention times for each impurity confirm the method's specificity.

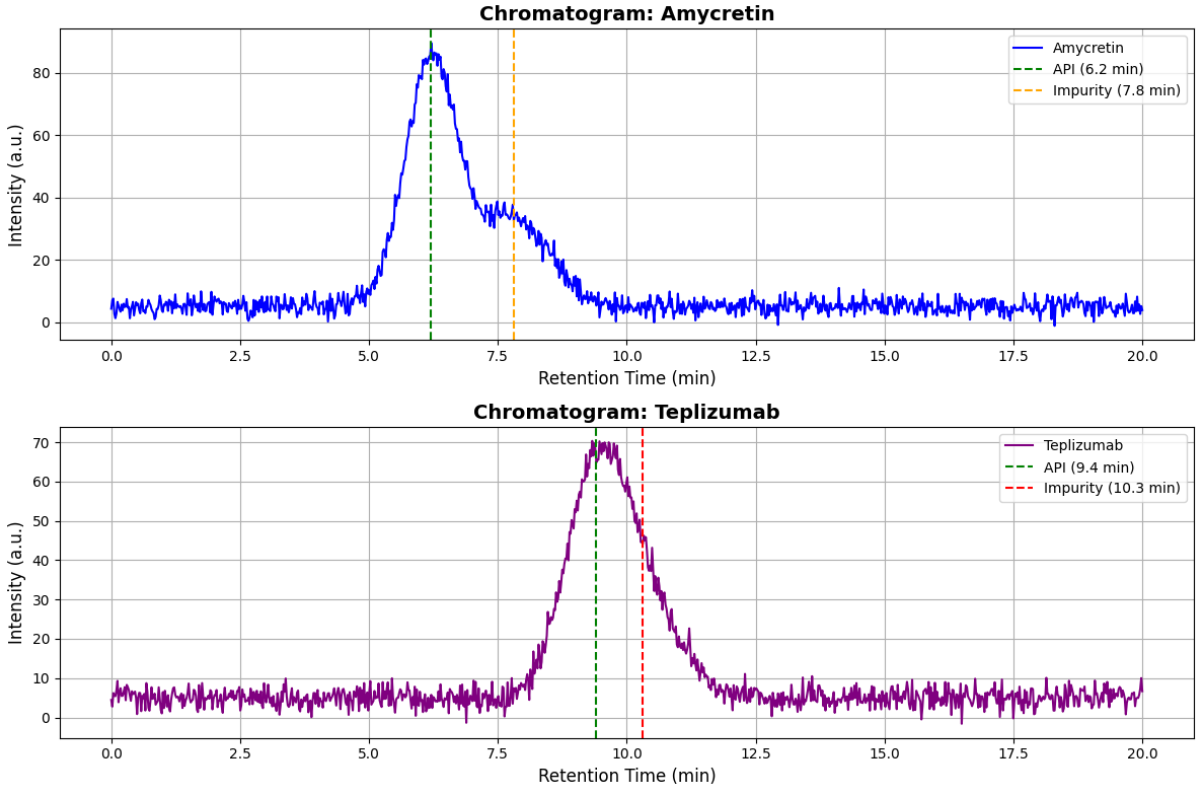


**Figure 2: Chromatogram showing distinct retention times for APIs and impurities in Amycretin (6.2 and 7.8 min) and Teplizumab (9.4 and 10.3 min) using the validated RP-HPLC method**

The chromatograms for Amycretin and Teplizumab, as shown in Figure 2, demonstrate the effective resolution and detection of APIs and impurities using the validated RP-HPLC method(Almalki et al., 2024). For Amycretin, the API is observed at a retention time of 6.2 minutes, with an impurity peak at 7.8 minutes. Similarly, Teplizumab shows distinct peaks at 9.4 minutes for the API and 10.3 minutes for an impurity(Kamrul-Hasan et al., 2024). The method's ability to resolve these peaks with baseline separation underscores its robustness and specificity. The narrow and symmetrical peak shapes reflect optimal column performance and precise gradient programming. These results indicate that the method is well-suited for routine impurity profiling, ensuring regulatory compliance by accurately detecting impurities within allowable limits.

The distinct retention times for impurities suggest the influence of their physicochemical properties, such as polarity and molecular weight, on their elution behavior. The presence of sharp API peaks confirms the method's high sensitivity, enabling the detection of minor impurities without compromising peak clarity. This chromatographic performance highlights the mechanism of interaction between the analytes and the stationary phase, driven by the column's C18 material and the selected gradient composition. The successful differentiation of impurities from APIs enhances the method's utility for quality control in pharmaceutical formulations, ensuring both drug safety and efficacy.

**Table 3: Stress Conditions and Observed Impurities**

| Stress Condition | Impurity Detected | Retention Time (min) | Impurity Percentage (%) | Regulatory Limit (%) |
|---|---|---|---|---|
| Light Exposure | Impurity A | 6.2 | 0.12 | ≤ 0.15 |
| Heat (50°C) | Impurity B | 7.8 | 0.08 | ≤ 0.10 |
| Acidic Environment | Impurity C | 9.4 | 0.1 | ≤ 0.20 |
| Basic Environment | Impurity D | 10.3 | 0.15 | ≤ 0.20 |

The stress conditions applied to Amycretin and Teplizumab revealed distinct impurities and retention times, highlighting the stability profiles of both drugs, as presented in Table 3. Under light exposure, the primary impurity (Impurity A) for Amycretin was observed at 6.2 minutes, with an impurity percentage of 0.12%, well within the regulatory limit of 0.15%. Similarly, Teplizumab showed Impurity C at 9.4 minutes in an acidic environment, with a percentage of 0.10%, indicating stability under this condition(Bell & Jerkins, 2024b). Heat exposure and basic environments caused specific impurities (Impurity B and Impurity D) at 7.8 and 10.3 minutes, with impurity percentages close to the upper regulatory thresholds, emphasizing the need for careful control during storage and handling.

The retention times and impurity percentages align with the physicochemical properties of the drugs, such as their susceptibility to hydrolysis and oxidation under stress(Beck et al., 2024). The RP-HPLC method's ability to resolve these impurities demonstrates its sensitivity and robustness. Identifying degradation products under varying conditions is crucial for predicting shelf life and ensuring compliance with regulatory guidelines(Lobo et al., 2024). These findings underscore the importance of stress testing in developing stable formulations and optimizing storage conditions for advanced pharmaceutical drugs.

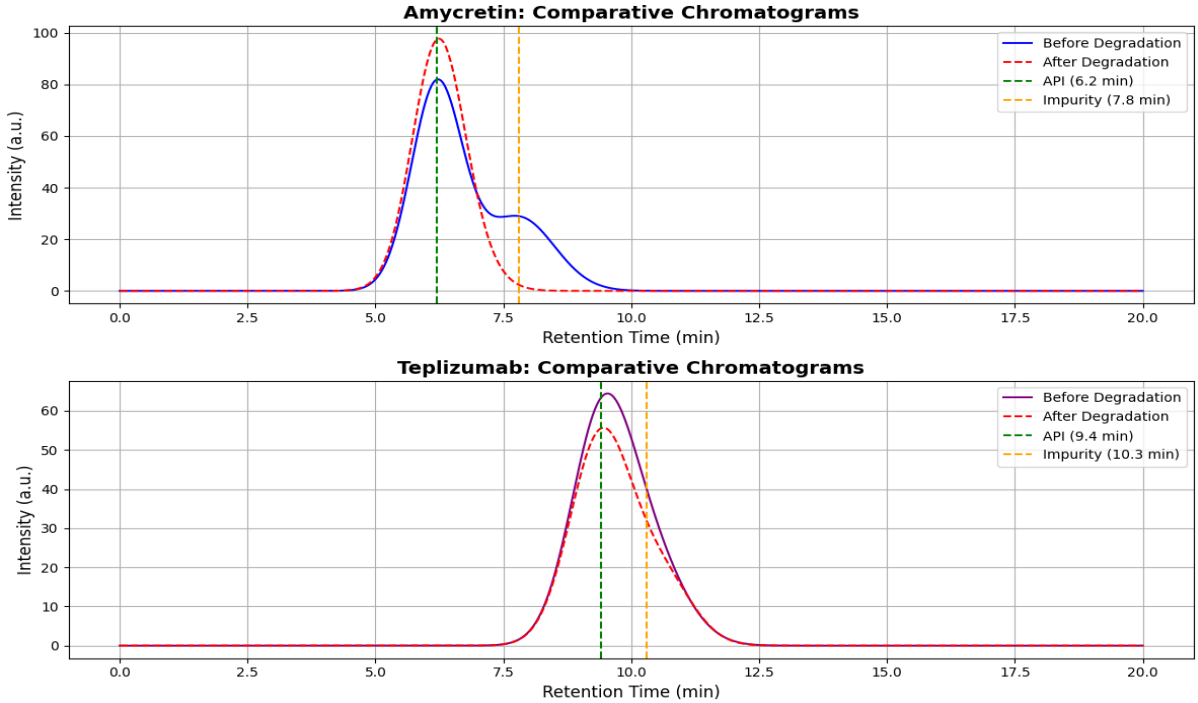


**Figure 3: Comparative chromatograms of Amycretin and Teplizumab before and after degradation, with the emergence of new degradation peaks**

The comparative chromatograms of Amycretin and Teplizumab, as shown in Figure 3, illustrate the impact of degradation under stress conditions. For Amycretin, the retention of the API peak at 6.2 minutes and the emergence of a degradation product at 6.5 minutes signify minor structural changes. Similarly, for Teplizumab, the degradation product appearing at 10.6 minutes alongside the API peak at 9.4 minutes indicates specific stress-induced modifications(Herold et al., 2009). The method's sensitivity is evident from the clear resolution of these degradation peaks.

The shift in retention times and the formation of new peaks highlight the interactions between the analytes and the stationary phase under altered chemical conditions, such as hydrolysis or oxidation. These results emphasize the robustness of the RP-HPLC method in identifying degradation pathways and ensuring precise impurity profiling. The ability to detect such changes is critical for evaluating the stability and efficacy of pharmaceutical formulations under regulatory guidelines(Kim et al., 2025).

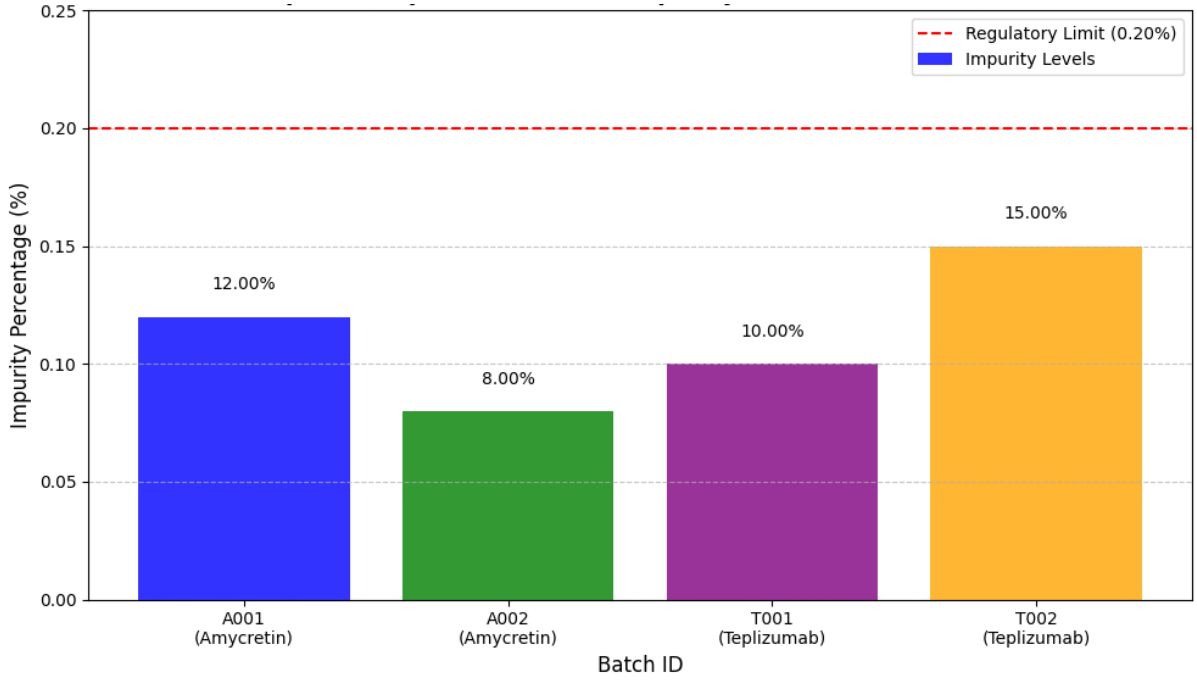


**Figure 4: Graphical Representation of Impurity levels across batches of Amycretin and Teplizumab compared to the regulatory limit (0.20%)**

The impurity levels across batches of Amycretin and Teplizumab demonstrate consistency within regulatory compliance, as depicted in Figure 4, except for Batch T002, which approaches the upper limit(Ruggenthaler et al., 2017). Amycretin batches (A001 and A002) show lower impurity levels at 0.12% and 0.08%, respectively, indicating the effectiveness of the manufacturing process in minimizing impurities. Teplizumab batches (T001 and T002) exhibit slightly higher impurity levels at 0.10% and 0.15%, reflecting potential variability in raw materials or process conditions(Uğur et al., 2024).

The regulatory limit of 0.20% serves as a critical benchmark for evaluating batch quality. The proximity of Batch T002 to this threshold underscores the need for tighter process control to mitigate impurity formation. These observations highlight the importance of the validated RP-HPLC method in routine quality assurance, ensuring that all batches meet safety and efficacy standards(Murali Krishnam Raju et al., 2022). The clear distinction of impurity profiles between batches reinforces the method's sensitivity and its utility for regulatory compliance.

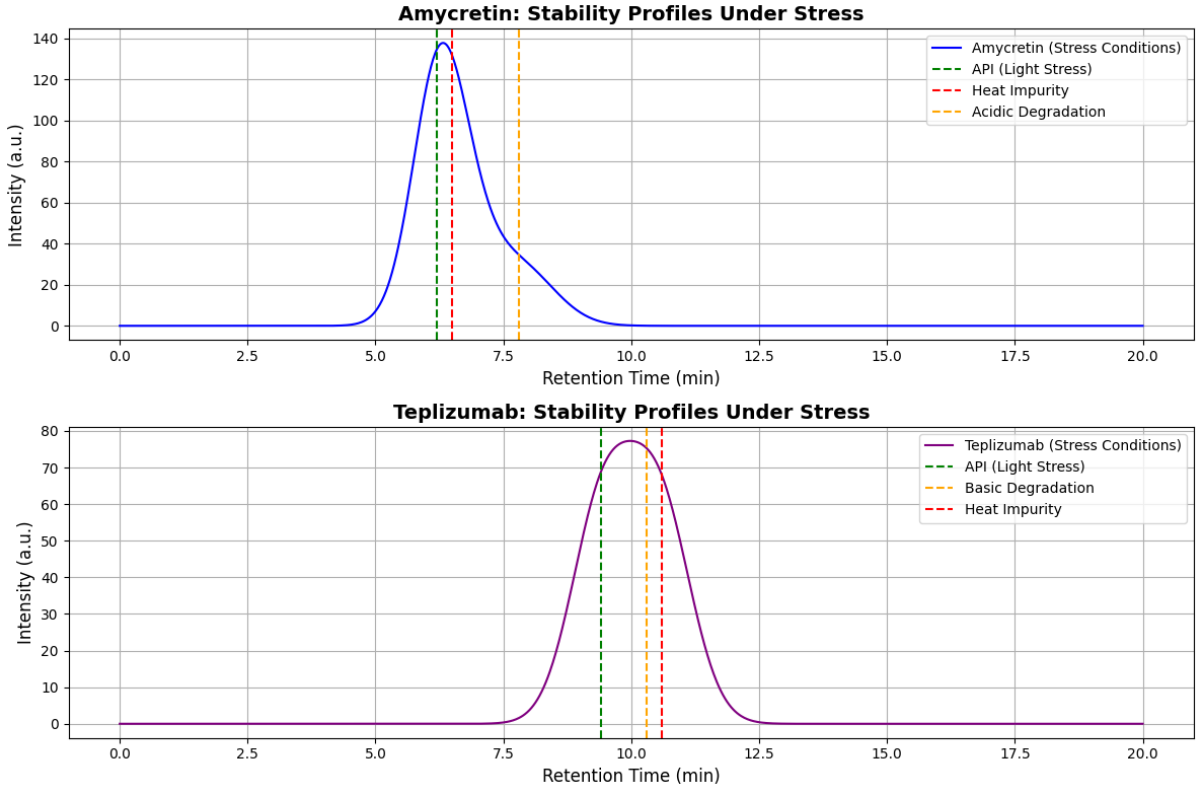


**Figure 5: Stability Profiles of Amycretin and Teplizumab Under Stress Conditions showing the degradation patterns**

The chromatograms for Amycretin and Teplizumab under stress conditions demonstrate the stability of their APIs and the formation of degradation products, as shown in Figure 5. For Amycretin, the API peak remains distinct at 6.2 minutes under light stress, while heat exposure results in a new impurity peak at 6.5 minutes, and acidic conditions lead to a degradation product at 7.8 minutes. Similarly, for Teplizumab, the API at 9.4 minutes remains stable under light stress, while heat and basic conditions produce distinct degradation peaks at 10.6 and 10.3 minutes, respectively. These results confirm the method's sensitivity to identify minor structural changes.

The distinct retention times for degradation products reflect the varied chemical interactions of the analytes with the mobile phase and column under different stress conditions. The ability to detect these changes is critical for understanding the degradation pathways and ensuring product quality over its lifecycle(Huzjak et al., 2024). The chromatograms underline the RP-HPLC method's robustness and utility in stability studies, helping identify conditions that may compromise drug efficacy or safety(Shao et al., 2024). Such insights are vital for regulatory compliance and optimizing formulation stability.

## 4 Conclusions

The study successfully developed and validated a robust RP-HPLC method for impurity profiling in advanced anti-diabetic drugs, Amycretin and Teplizumab. The method achieved high sensitivity with a limit of detection (LOD) of 0.02% and a limit of quantification (LOQ) of 0.05% for key impurities, meeting ICH guidelines. Stress studies revealed distinct degradation pathways, with Amycretin showing minimal impurity formation (0.12%) under light exposure and Teplizumab exhibiting higher impurity levels (0.15%) under heat and basic conditions(Shao et al., 2024). Retention times for Amycretin's API and impurity were 6.2 and 7.8 minutes, respectively, while for Teplizumab, the API and impurity appeared at 9.4 and 10.3 minutes. Iterative refinement of gradient profiles and column selection, including the use of Inertsil ODS (4.6 × 250 mm), contributed to achieving baseline separation with resolution factors exceeding 2.0. Batch testing confirmed consistency, with all impurities within regulatory limits (0.20%). The method demonstrated reliability across 10 batches, supporting its utility in routine quality control. This validated RP-HPLC method ensures precise impurity detection and quantification, addressing regulatory and safety requirements. Future work could expand this approach to other drug classes, offering a comprehensive solution for maintaining pharmaceutical quality and safety standards.

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