# Challenges and Progress in Related Substance Method Development: A Review of Validation Protocols

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**Abstracts:** The detection and quantification of related substances, including impurities and degradation products, are crucial for ensuring the quality and safety of pharmaceutical products. The development of robust analytical methods for related substances has faced numerous challenges, ranging from the complexity of pharmaceutical formulations to stringent regulatory requirements. This review highlights the key hurdles in related substance method development, including issues related to sensitivity, selectivity, and matrix effects, as well as the need for stability-indicating capabilities. It also examines the progress made in the field, particularly through the introduction of advanced chromatographic techniques such as UHPLC and SFC, and the coupling of mass spectrometry for enhanced sensitivity and specificity. The review further explores the role of automated method development and the adoption of standardized validation protocols guided by regulatory frameworks, alongside the application of Quality by Design (QbD) principles to optimize and ensure the robustness of analytical methods. Despite significant advancements, continuous monitoring, revalidation, and adaptation to emerging impurities remain critical for maintaining the reliability of these methods in pharmaceutical quality control.

**Keywords:** Related Substances, Method Development, UHPLC, SFC, Mass Spectrometry, Validation Protocols, Quality by Design, Pharmaceutical Analysis, Impurities, Regulatory Guidelines

## 1. INTRODUCTION

In the pharmaceutical industry, the identification and quantification of related substances—often referred to as impurities—are paramount to ensuring the safety, efficacy, and quality of pharmaceutical products. Related substances can arise from various sources, including degradation products, process-related impurities, and interaction with excipients. Their presence, even in trace amounts, can significantly impact the therapeutic performance and safety profile of the final product [1]. The development of robust analytical methods to accurately detect and quantify these impurities is not just a scientific challenge but also a regulatory imperative. Regulatory agencies like the FDA, EMA, and ICH have established stringent guidelines for impurity analysis, underscoring its critical role in pharmaceutical quality control [2,3]. These guidelines mandate that pharmaceutical companies not only identify and quantify impurities but also validate the analytical methods used to ensure they are precise, accurate, and reliable [4].

This review article aims to provide a comprehensive overview of the challenges faced in the development of related substance analytical methods and the significant advancements that have been made in this field. By examining both the hurdles and the innovations, this review underscores the importance of continual improvement in analytical methodologies to meet the ever-evolving demands of regulatory bodies and to safeguard patient health

## 2. Challenges in Related Substance Method Development

The development of analytical methods for the detection and quantification of related substances in pharmaceutical products is fraught with numerous challenges. These challenges arise from the inherent complexity of pharmaceutical formulations, the stringent requirements set by regulatory authorities, and the need for methods that can reliably differentiate between various types of impurities [5-9].

**Complexity of Pharmaceutical Formulations:** Pharmaceutical products often comprise a complex mixture of the active pharmaceutical ingredient (API), excipients, and other additives, each of which can interfere with the accurate detection of impurities. The presence of multiple components can lead to co-elution in chromatographic methods, making it difficult to separate and accurately quantify impurities. This complexity necessitates the development of highly selective methods that can isolate and analyze the related substances without interference from other formulation components.

**Sensitivity and Selectivity:** One of the primary challenges in related substance analysis is achieving the required sensitivity and selectivity. Impurities are typically present at very low concentrations compared to the API, making their detection and quantification challenging. Analytical methods must be sensitive enough to detect these trace levels of impurities while being selective enough to distinguish them from the API and other excipients. This balance between sensitivity and selectivity is often difficult to achieve, especially in complex matrices.

**Matrix Effects:** The sample matrix can significantly influence the performance of an analytical method, leading to inaccurate results. Matrix effects, such as ion suppression or enhancement in mass spectrometry, can alter the response of the analytes and affect the accuracy and precision of the method. Overcoming matrix effects requires thorough method development and validation, including the use of appropriate sample preparation techniques and the selection of suitable internal standards to compensate for these effects.

**Stability-Indicating Capability:** A critical aspect of related substance method development is the ability to distinguish between impurities that arise from degradation and those that are present as process-related substances. Stability-indicating methods are essential for ensuring that the method can detect and quantify impurities that result from the degradation of the API under various conditions, such as exposure to light, heat, or humidity. Developing such methods requires a deep understanding of the degradation pathways of the API and the potential impurities that may form.

**Regulatory Requirements:** Adherence to stringent regulatory guidelines is another significant challenge in related substance method development. Regulatory agencies such as the FDA, ICH, and EMA require that pharmaceutical companies develop and validate analytical methods that are robust, accurate, and reliable. These methods must be capable of consistently detecting and quantifying impurities at specified levels, ensuring that the drug product meets the required safety and efficacy standards. Compliance with these guidelines involves extensive method validation, including the assessment of parameters such as specificity, linearity, accuracy, precision, robustness, and limits of detection and quantification

**Table 1: Challenges in Related Substance Method Development**

| Challenge | Description |
|---|---|
| **Complexity of Pharmaceutical Formulations** | Pharmaceutical products contain multiple components (API, excipients, additives) that can interfere with the accurate detection of impurities, leading to challenges in separation and quantification. |
| **Sensitivity and Selectivity** | Methods must achieve high sensitivity to detect low levels of impurities while maintaining selectivity to distinguish impurities from the API and other formulation components. |
| **Matrix Effects** | The sample matrix can alter the analytical response, leading to inaccurate results. Overcoming matrix effects requires careful method development, sample preparation, and validation. |
| **Stability-Indicating Capability** | Methods must distinguish between impurities arising from degradation and those present as process-related substances, requiring a deep understanding of degradation pathways. |
| **Regulatory Requirements** | Methods must adhere to stringent regulatory guidelines (FDA, ICH, EMA) requiring robustness, accuracy, and reliability in detecting and quantifying impurities. Extensive validation is necessary. |

## 3. Progress in Method Development

### 3.1 Advanced Chromatographic Techniques

#### Introduction

The continuous demand for improved analytical methods in pharmaceutical research has led to the development of advanced chromatographic techniques such as Ultra-High-Performance Liquid Chromatography (UHPLC) and Supercritical Fluid Chromatography (SFC). These techniques have significantly elevated the standards of separation efficiency and detection sensitivity, particularly in the context of related substance analysis [10-12].

#### Principle

**UHPLC** operates on the same basic principles as traditional HPLC but with significant advancements in pressure capacity. The core idea is to utilize smaller particle sizes in the stationary phase, which provides greater surface area and improved interaction with analytes. This results in sharper peaks and faster elution times, even under high pressure.

**SFC** utilizes supercritical fluids, typically carbon dioxide, as the mobile phase. The unique properties of supercritical fluids, which exhibit both gas-like diffusion and liquid-like solvation, enable rapid and efficient separation of analytes. The ability to fine-tune the mobile phase properties by adjusting pressure and temperature further enhances separation performance.

#### Instrumentation

**UHPLC Systems**: The main components include high-pressure pumps capable of delivering mobile phases at pressures up to 15,000 psi, a column packed with sub-2-micron particles, an auto-sampler, a detector (commonly UV or PDA), and data acquisition software. The system is designed to withstand the high pressures associated with UHPLC and to ensure reproducibility and precision in analysis.

**SFC Systems**: A typical SFC setup consists of a CO2 delivery system, high-pressure pumps, a column oven, a packed or capillary column designed for supercritical conditions, a back pressure regulator, and a detector (often UV or MS). The system must maintain the CO2 in its supercritical state throughout the separation process, necessitating precise control of temperature and pressure.

#### Procedure

**UHPLC**: The sample is introduced into the system through an auto-sampler and carried through the column by the high-pressure mobile phase. As the analytes pass through the column, they interact with the stationary phase, leading to separation based on their chemical properties. The separated analytes are then detected, usually by a UV detector, and data is recorded.

**SFC**: The sample is injected into the supercritical CO2 mobile phase, often modified with a small amount of organic solvent (modifier) to enhance solubility and separation. The analytes are separated as they pass through the supercritical fluid in the column and are detected at the end of the column. The use of a back pressure regulator ensures the CO2 remains in a supercritical state throughout the process.

#### Applications

**UHPLC**: This technique is widely used in the pharmaceutical industry for the rapid analysis of related substances, degradation products, and impurities. Its high resolution and speed make it ideal for high-throughput environments, where time and accuracy are critical. UHPLC is also employed in the analysis of complex biological samples, where precise quantification of low-concentration analytes is required.

**SFC**: SFC is particularly useful for the separation of chiral compounds, lipids, and other non-polar or slightly polar substances that are difficult to separate using traditional liquid chromatography. It is increasingly used in the pharmaceutical industry for the purification of drug compounds and the analysis of complex mixtures where traditional methods fall short.

### 3.2 Mass Spectrometry Coupling

#### Introduction

The coupling of chromatographic methods with mass spectrometry (MS) has revolutionized the analysis of related substances, enabling the identification and quantification of impurities at very low concentrations. Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) has become a benchmark technique in the pharmaceutical industry due to its high sensitivity, specificity, and capability for structural elucidation of complex analytes[13-16].

#### Principle

Mass spectrometry works on the principle of ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios. When coupled with chromatography, the separated analytes are introduced into the mass spectrometer, where they are ionized, typically by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The ions are then separated based on their mass-to-charge ratios and detected, allowing for both qualitative and quantitative analysis.

#### Instrumentation

**Chromatographic Interface**: The interface between the chromatography system (typically HPLC or UHPLC) and the mass spectrometer is crucial. It must efficiently transfer the analytes from the chromatographic system into the ion source of the mass spectrometer without compromising the separation achieved during chromatography.

**Ion Source**: The ion source, such as ESI or APCI, ionizes the analytes as they enter the mass spectrometer. The choice of ionization method depends on the nature of the analytes and the sensitivity required for the analysis.

**Mass Analyzer**: The mass analyzer (e.g., quadrupole, time-of-flight, or ion trap) separates ions based on their mass-to-charge ratio. In tandem mass spectrometry (MS/MS), multiple stages of mass analysis are used to provide structural information about the analytes.

**Detector**: The detector measures the intensity of the ions, producing a mass spectrum that represents the distribution of ions by mass-to-charge ratio. This data is used to identify and quantify the analytes.

#### Procedure

In LC-MS/MS, the sample is first separated by the chromatographic system. The eluted compounds are then directed into the mass spectrometer, where they are ionized, analyzed, and detected. The resulting mass spectra are interpreted to identify and quantify the analytes. In MS/MS mode, selected precursor ions are further fragmented to produce product ions, which are analyzed to provide detailed structural information.

#### Applications

**Quantification of Trace Impurities**: LC-MS/MS is widely used for the quantification of trace impurities and degradation products in pharmaceutical formulations. Its high sensitivity allows for the detection of impurities at concentrations as low as parts per billion (ppb).

**Structural Elucidation**: The ability to perform MS/MS makes LC-MS/MS an invaluable tool for structural elucidation, enabling researchers to determine the molecular structure of unknown impurities.

**Bioanalytical Applications**: LC-MS/MS is also extensively used in pharmacokinetics and bioanalysis, where it is employed to measure drug concentrations in biological matrices such as plasma or urine, providing critical data for drug development and therapeutic monitoring.

### 3.3 Automated Method Development

Automated method development has transformed the landscape of chromatographic analysis, particularly in the context of related substance detection. Traditionally, method development was a labour-intensive and time-consuming process, requiring extensive trial and error to identify optimal chromatographic conditions. However, with the advent of automated tools and software, this process has become significantly more efficient, allowing for the rapid optimization of chromatographic methods [17].

Automated method development tools leverage advanced algorithms and machine learning techniques to predict the optimal chromatographic conditions based on the physicochemical properties of the analytes. These tools are capable of evaluating a wide range of parameters, including mobile phase composition, pH, column selection, flow rate, temperature, and detection wavelength. By simulating various conditions and assessing their impact on separation efficiency, these tools can quickly identify the most suitable parameters for achieving the desired separation and detection criteria [18,19].

Automated method development platforms are integrated into chromatographic systems, often linked to data acquisition software that controls the instrument and collects analytical data. The software is equipped with databases containing information on various stationary phases, mobile phases, and their interactions with different classes of compounds. The user inputs the known properties of the analytes, such as molecular weight, polarity, and pKa, and the software generates a series of potential chromatographic conditions. The system can then automatically run these conditions, collecting and analyzing the resulting data to identify the optimal method [20].

### 3.4 Validation Protocols

#### Introduction

The validation of analytical methods is a cornerstone of pharmaceutical analysis, ensuring that methods are reliable, reproducible, and fit for their intended purpose. Over the years, significant progress has been made in the standardization of validation protocols, mainly through the guidelines provided by the International Council for Harmonisation (ICH). These protocols have brought consistency and rigor to the validation process, making it possible to achieve high accuracy, precision, and robustness in related substance analysis [21-29].

The validation parameters referred from the work of Anil Kumar Tallam emphasize a structured approach to method validation, ensuring reliability and regulatory compliance in the analytical process. The following parameters were specifically highlighted and applied in the manuscript

#### System Suitability

System suitability tests (SSTs) are performed to ensure that the analytical system functions correctly before and during the analysis. These tests involve using reference standards and system suitability solutions to evaluate critical performance criteria such as resolution, tailing factor, theoretical plate number, and retention time consistency. SSTs are essential to method validation as they confirm that the chromatographic system can produce reliable results.

**Precision**

Precision refers to the consistency of the method when repeated under identical conditions. It is typically assessed at three levels:

**Repeatability**: The precision of the method under the same conditions over a short period, usually within a single day (intra-day precision).

**Intermediate Precision**: The precision of the method when performed on different days, by different analysts, and using different equipment (inter-day precision).

**Reproducibility**: The precision of the method when performed in different laboratories, often assessed during method transfer studies.

Precision is evaluated by analyzing multiple replicates of a sample and calculating the relative standard deviation (RSD) of the results.

**Accuracy**

Accuracy refers to the closeness of the measured values to the true value or the accepted reference value. It is evaluated by spiking known amounts of the analyte into the matrix and comparing the measured concentrations with the theoretical concentrations. Accuracy is typically expressed as a percentage recovery of the spiked analyte.

**Linearity**

Linearity assesses the method's ability to produce results that are directly proportional to the concentration of the analyte within a specified range. It is evaluated by analyzing samples at different concentrations and plotting the response against concentration. The correlation coefficient ($R^2$) is used to assess the method's linearity, with values close to 1 indicating a strong linear relationship.

**Specificity**

Specificity refers to the method's ability to measure the analyte of interest in the presence of other components, such as impurities, degradation products, and matrix components. Specificity is assessed by analyzing samples containing potential interfering substances and demonstrating that these substances do not affect the quantification of the analyte.

**Robustness**

Robustness evaluates the method's ability to remain unaffected by small, deliberate changes in method parameters, such as pH, mobile phase composition, flow rate, and temperature. Robustness testing ensures the method can produce reliable results under slightly varied conditions, which is crucial for its practical application.

**Detection and Quantitation Limits**

The detection limit (LOD) is the lowest concentration of the analyte that can be detected but not necessarily quantified. In contrast, the quantitation limit (LOQ) is the lowest concentration that can be quantified with acceptable precision and accuracy. These limits are determined through statistical analysis of the response data and are critical for ensuring that the method is sensitive enough to detect and quantify trace impurities.

**Applications**

Validated methods are used across various stages of pharmaceutical development and manufacturing, including:

**Quality Control**: Ensuring that pharmaceutical products meet the required specifications for purity, potency, and safety.

**Regulatory Submissions**: Providing validated methods as part of regulatory submissions to demonstrate the quality of the product.

**Stability Testing**: Using validated methods to monitor the stability of pharmaceutical products over time, ensuring that they remain within the specified quality standards throughout their shelf life

### 3.5 Quality by Design (QbD)

#### Introduction

Quality by Design (QbD) is a systematic approach to pharmaceutical development that emphasizes the importance of designing quality into the product and process from the outset. In the context of analytical method development, QbD principles have been adopted to enhance the robustness and reliability of methods for related substance analysis. By understanding the critical quality attributes (CQAs) and the relationship between method variables and performance, QbD allows for the systematic optimization of methods to ensure consistent and reliable results [30-34].

#### Principle

QbD in method development involves a comprehensive understanding of the analytical method's performance characteristics and the factors that influence them. This approach is based on the following key components:

**Critical Quality Attributes (CQAs)**: These are the specific attributes of the analytical method that must be controlled to ensure that it meets the required quality standards. CQAs may include parameters such as resolution, peak symmetry, detection limits, and accuracy.

**Risk Assessment**: QbD involves a thorough risk assessment to identify the potential sources of variability that could affect the method's performance. By identifying and controlling these risks, the method can be optimized to produce reliable results under a wide range of conditions.

**Design of Experiments (DoE)**: QbD utilizes Design of Experiments (DoE) to systematically study the impact of various method parameters on the CQAs. DoE allows for the identification of optimal conditions that maximize the method's robustness while minimizing the risk of failure.

**Control Strategy**: A control strategy is developed based on the results of the DoE studies, outlining the acceptable ranges for critical method parameters and the steps to be taken to ensure that the method remains within these ranges during routine use.

#### Procedure

1. **Definition of CQAs**: The first step in QbD is to define the critical quality attributes of the method. These attributes are determined based on the intended use of the method and the regulatory requirements.

2. **Risk Assessment and Identification of Critical Method Parameters (CMPs)**: A risk assessment is conducted to identify the critical method parameters (CMPs) that have the most significant impact on the CQAs. This step involves using tools such as Ishikawa diagrams or Failure Mode and Effects Analysis (FMEA) to assess the risks associated with each parameter.

3. **Design of Experiments (DoE)**: A DoE study is conducted to explore the relationships between the CMPs and the CQAs. The study involves systematically varying the CMPs and analyzing the resulting data to identify the optimal conditions for the method.

4. **Method Optimization**: Based on the DoE results, the method is optimized to achieve the desired performance. This may involve fine-tuning the mobile phase composition, adjusting the column temperature, or modifying the gradient program.

5. **Control Strategy Implementation**: A control strategy is developed to ensure the method remains robust and reliable during routine use. This strategy includes monitoring key method parameters, establishing acceptance criteria, and implementing corrective actions if deviations occur.

In the manuscript, the reference article by Mohana Vamsi Nuli, Ramanjaneyulu Seemaladinne, and Anil Kumar Tallam was utilized for the application of Analytical Quality by Design (AQbD). By integrating QbD, the method development process systematically evaluates critical quality attributes (CQAs) and critical method parameters (CMPs), ensuring optimal performance and regulatory compliance. This approach helps achieve consistent and reliable results, especially in pharmaceutical analysis, as highlighted by the authors.

**Applications**

QbD has become an integral part of method development in the pharmaceutical industry, particularly in the following areas:

**Method Robustness**: Ensuring that analytical methods are robust and capable of producing reliable results under various conditions.

**Regulatory Compliance**: Meeting regulatory expectations for method validation and ensuring that methods are developed in a systematic and scientifically sound manner.

**Continuous Improvement**: Facilitating continuous improvement of analytical methods by providing a framework for ongoing monitoring and optimization.

**Table 2: Automated Method Development**

| Parameter | Description |
|---|---|
| **Input of Analyte Information** | User provides details about the analytes such as chemical structure, molecular weight, and known chromatographic behavior. |
| **Parameter Exploration** | Software simulates different chromatographic conditions using algorithms to predict optimal separation. |
| **Experimental Execution** | The system automatically runs chromatographic conditions, collecting and analyzing data on separation efficiency. |
| **Data Analysis and Optimization** | Analyzes collected data to identify optimal chromatographic conditions, further refining parameters through iterations. |
| **Method Finalization** | Optimized method is validated and documented for routine use. |

**Table 3: Validation Protocols**

| Parameter | Description |
|---|---|
| **System Suitability** | Ensures the chromatographic system functions correctly, evaluating performance criteria such as resolution and retention time. |
| **Precision** | Assesses the consistency of the method under identical conditions, including repeatability, intermediate precision, and reproducibility. |
| **Accuracy** | Evaluates the closeness of measured values to true values by spiking known amounts of analyte into the matrix. |
| **Linearity** | Assesses the method's ability to produce results proportional to the analyte concentration, using correlation coefficients ($R^2$). |

| | |
|---|---|
| **Specificity** | Measures the method's ability to quantify the analyte in the presence of impurities, degradation products, and matrix components. |
| **Robustness** | Evaluates the method's resistance to small, deliberate changes in parameters like pH, mobile phase composition, and temperature. |
| **Detection and Quantitation Limits** | Determines the lowest concentration of analyte that can be detected (LOD) and quantified (LOQ) with acceptable accuracy. |

**Table 3: Quality by Design (QbD)**

| Component | Description |
|---|---|
| **Critical Quality Attributes (CQAs)** | Key attributes of the method that must be controlled to ensure it meets required quality standards. |
| **Risk Assessment** | Identifies potential sources of variability that could affect method performance, using tools like FMEA or Ishikawa diagrams. |
| **Design of Experiments (DoE)** | Systematic study of the impact of method parameters on CQAs, leading to identification of optimal conditions. |
| **Method Optimization** | Fine-tuning method parameters based on DoE results to achieve desired performance. |
| **Control Strategy Implementation** | Developing a strategy to monitor and control key method parameters during routine use, ensuring method robustness. |

## 4. Challenges in Validation Protocols

Challenges in validation protocols often arise from the need to ensure stringent accuracy, precision, and reproducibility across different laboratory settings [35-36]

### Stringent Accuracy and Precision Requirements

One of the primary challenges in method validation is meeting stringent accuracy and precision requirements, particularly when dealing with low concentrations of related substances. Pharmaceutical products often contain trace levels of impurities that must be detected and quantified with a high degree of confidence. Achieving this level of precision requires rigorous testing, including multiple replicates and the use of reference standards, to ensure that the method consistently produces accurate results. However, the complexity of pharmaceutical matrices, which may contain active ingredients, excipients, and degradation products, can interfere with the analysis, making it difficult to achieve the necessary level of accuracy. As a result, extensive method optimization is often required, including careful selection of chromatographic conditions, detection techniques, and sample preparation methods.

### Reproducibility Across Laboratories

Regulatory guidelines, such as those from the International Council for Harmonisation (ICH), emphasize the importance of reproducibility across different laboratories. This ensures that validated methods can be reliably used in various settings, including during regulatory submissions, product development, and quality control. However, achieving reproducibility can be challenging due to variability in laboratory equipment, environmental conditions, reagent quality, and operator skill. For example, differences in instrument calibration, temperature control, and even sample handling procedures can lead to variations in method performance. To address this, inter-laboratory studies, or ruggedness testing, are often conducted to evaluate the method's robustness across different settings. These studies help to identify any potential sources of variability and ensure that the method produces consistent results regardless of where it is applied.

### Continuous Monitoring and Revalidation

Validation does not end once a method has been approved for use; continuous monitoring and periodic revalidation are essential to ensure that the method remains suitable over time. Regulatory agencies require pharmaceutical companies to implement ongoing monitoring programs to assess the performance of validated methods during routine use. Any changes in raw materials, manufacturing processes, or equipment may affect the method's performance, necessitating revalidation to confirm its continued suitability. Additionally, long-term stability studies may reveal new degradation products or impurities that were not initially present, requiring modifications to the

method. This ongoing need for monitoring and revalidation adds to the complexity and cost of maintaining validated methods, especially in industries where products undergo frequent reformulation or process changes.

### Emerging Impurities

The pharmaceutical industry is continuously evolving, with new synthetic routes, formulations, and manufacturing processes being developed to meet the demands of modern medicine. These innovations often introduce new impurities that were not previously encountered, presenting a significant challenge for method validation. In some cases, the new impurities may be structurally similar to the active ingredient, making them difficult to detect and quantify using existing methods. This requires the development of new analytical techniques or modifications to existing methods to ensure that emerging impurities are properly identified and controlled. The detection of genotoxic impurities, which pose a significant risk even at trace levels, has become a particular focus of regulatory agencies, further driving the need for advanced analytical methods capable of detecting extremely low concentrations of these compounds.

## CONCLUSION

In conclusion, the development and validation of methods for detecting related substances remain an essential yet challenging aspect of pharmaceutical quality control. While advancements in chromatographic techniques and the integration of mass spectrometry have improved sensitivity and specificity, the complexity of formulations and the need for continuous method adaptation present ongoing hurdles. The adoption of automated tools and standardized validation protocols, particularly underpinned by QbD principles, has significantly enhanced the efficiency and robustness of method development. However, the dynamic nature of pharmaceutical manufacturing, along with the emergence of new impurities, necessitates continuous monitoring, revalidation, and innovation in analytical methodologies. Addressing these challenges will be crucial to ensuring the safety and efficacy of pharmaceutical products in an increasingly complex regulatory environment.

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