RESEARCH ARTICLE

A HOLISTIC APPROACH TO HPLC TECHNIQUEFOR ADDRESSING PROCESS IMPURITIES: REVIEW ONMETHOD DEVELOPMENT, OPTIMIZATION, AND VALIDATION

P. Chandrashekhar Reddy[1], G. Sampath Kumar Reddy[2] and B. Jainendra Kumar[3]
1. Department of Chemistry, Anurag University, Hyderabad, India.
2. Analytical Research and Development, Aurore Life Sciences Private Limited, Hyderabad, India.
3. Department of Pharmacy, Anurag University, Hyderabad, India.

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Manuscript Info	*Abstract*
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	High-Performance Liquid Chromatography (HPLC) has become an indispensable analytical tool in pharmaceutical, environmental, and food sciences due to its precision, sensitivity, and versatility. A holistic approach to HPLC method development, optimization, and validation ensures reliable and reproducible results while meeting regulatory requirements. This review outlines the critical steps in HPLC method development, emphasizing the importance of understanding analyte properties, selecting appropriate chromatographic conditions, and fine-tuning parameters for optimal performance. Key aspects of method optimization, such as mobile phase composition, column selection, flow rate, and pH, are discussed to enhance resolution and reduce analysis time. Additionally, the article delves into the rigorous validation process, which includes assessing accuracy, precision, specificity, linearity, and robustness in compliance with International Council for Harmonisation (ICH) guidelines. By integrating systematic method development, experimental optimization, and thorough validation, this holistic framework ensures efficient and high-quality analytical methods, driving advancements in research and quality assurance across various industries.

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Introduction:-

High-Performance Liquid Chromatography (HPLC) remains a cornerstone analytical technique in the pharmaceutical industry, vital for detecting contaminants such as process-related impurities, degradation products, and genotoxic contaminants (GTCs), including nitrosamines. Regulatory frameworks like ICH M7 [1] stress the importance of controlling these impurities, which must be monitored at trace levels to minimize potential carcinogenic risks and ensure pharmaceutical safety. Recent advancements in HPLC technology have significantly enhanced its ability to detect and quantify these contaminants with greater sensitivity and precision. One major development is the rise of ultra-high-performance liquid chromatography (UHPLC), which utilizes smaller particle sizes and higher column pressures, providing faster analysis with superior resolution compared to traditional HPLC [2]. This advancement is crucial for identifying trace-level impurities in complex matrices.

Additionally, the integration of mass spectrometry (MS) with HPLC (HPLC-MS/MS) has revolutionized impurity analysis. The enhanced sensitivity of HPLC-MS/MS enables the detection of impurities at ultra-low concentrations,

Corresponding Author:-P. Chandrashekhar Reddy
Address**:-**Department of Chemistry, Anurag University, Hyderabad, India.

facilitating the identification of both known and unknown contaminants. HPLC-MS/MS, a tandem mass spectrometry technique, offers further improvements in structural elucidation, aiding the identification of degradation products and their sources [3]. Innovations in stationary phase technology, such as advanced reversed-phase columns, have also improved the separation of complex mixtures, essential for distinguishing closely eluting impurities and isomer from active pharmaceutical ingredients [4].

Furthermore, the application of machine learning and AI in HPLC method optimization is gaining traction. These technologies predict optimal chromatographic conditions, improving method robustness and reproducibility [5]. Collectively, these advancements in HPLC technology ensure more efficient, sensitive, and reliable impurity profiling, meeting stringent regulatory requirements and improving pharmaceutical safety.

Main Text
Introduction to HPLC
High-Performance Liquid Chromatography (HPLC) is a chromatographic technique that separates compounds based on interactions with a stationary phase, influenced by factors such as polarity, size, and structure [6]. It operates under high pressure, which significantly enhances resolution and sensitivity, making it highly effective for complex sample analysis . HPLC is widely utilized in pharmaceuticals, biotechnology, food safety, and environmental monitoring [7], where it provides high-throughput, reproducible, and precise results . In the pharmaceutical industry, HPLC is critical for quantifying active pharmaceutical ingredients (APIs), impurities, degradation products, and genotoxic contaminants (GTCs), such as nitrosamines, The integration of mass spectrometry (LC-MS/MS) with HPLC offers significant improvements in sensitivity, enabling the detection of trace-level contaminants [8], which is crucial for ensuring drug safety and regulatory compliance [1]. Additionally, innovations such as core-shell and monolithic columns have contributed to improved separation efficiency, allowing the analysis of complex samples while meeting stringent regulatory standards.

However, despite these technological advancements, several challenges persist in current HPLC practices. The increasing complexity of pharmaceutical formulations, which may contain minute levels of impurities or degradation products, presents a challenge for detection using traditional HPLC methods, although core-shell and monolithic columns improve separation efficiency [10], they still have limitations in resolving highly similar compounds or trace-level contaminants . Furthermore, while the integration of LC-MS/MS enhances sensitivity, optimizing these systems for routine high-throughput analysis is still difficult, especially when handling large sample sets.

This review provides a holistic approach to overcoming these challenges through method development, optimization, and validation strategies that focus on enhancing the overall HPLC process. Recent innovations, such as hybrid columns, advanced detection techniques, and data-driven optimization tools, are discussed to offer solutions for addressing current limitations. The novelty of this study lies in its comprehensive examination of how these advancements can improve impurity profiling in pharmaceutical analysis, ensuring compliance with evolving regulatory standards and enhancing drug safety [1].

HPLC in Overcoming Analytical Obstacles: Perspectives and Strategies
The adoption of HPLC in pharmaceutical and industrial quality control is constrained by a series of analytical and technical challenges, including:

Identification Quantification and Profiling of Genotoxic Impurities (GTIs) in Pharmaceutical Compounds:
Genotoxic impurities, which have the potential to induce DNA damage and are regarded as potential carcinogens, must be detected at exceedingly low concentrations (ppm) levels [8]. Regulatory frameworks [11-12], underscore the imperative to utilize highly sensitive and precise analytical methodologies, such as HPLC, for the detection and quantification of these impurities.

Advanced techniques for the dissection and examination of multifaceted sample matrices.:
Advanced techniques for the dissection and examination of complex sample matrices using High-Performance Liquid Chromatography (HPLC) include precise sample preparation methods such as solid-phase extraction (SPE), which aids in isolating target analytes [1]. The application of gradient elution enhances separation efficiency, particularly for complex samples containing compounds with diverse polarities [2]. Coupling HPLC with highly sensitive detectors, such as mass spectrometry (MS) or UV-Vis, ensures high specificity and sensitivity, which is crucial for detecting

trace impurities. The use of specialized columns and automated sample injection systems further enhances resolution and reproducibility [4]. These innovations make HPLC a powerful analytical tool in diverse industries. The complexity of pharmaceutical products or drug substances can result in matrix effects that hinder accurate detection of genotoxic impurities (GTIs). To effectively separate target compounds from complex mixtures, advanced sample preparation techniques, such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE), are essential for isolating analytes of interest [12,13]

Innovation Demands:
Identifying genotoxic impurities (GTIs) in pharmaceuticals requires advanced instruments, such as High-Performance Liquid Chromatography (HPLC) coupled with Mass Spectrometry (LC-MS) and Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS), for precise trace-level analysis. These technologies provide high resolution and accuracy, ensuring compliance with regulatory standards [11]. Automated sample preparation systems enhance efficiency and minimize contamination risks [12,13]

Leading-edge methods for the quantification and characterization of genotoxic impurities and nitrosamines in pharmaceutical drug substances.
Leading-edge methods for the quantification and characterization of genotoxic impurities and nitrosamines in pharmaceutical drug substances are critical for ensuring the safety and efficacy of pharmaceutical products. Prominent examples include N-Nitroso dimethylamine (NDMA) and N-Nitrosodiethylamine (NDEA) [14]. These methods often employ highly sensitive techniques such as High-Performance Liquid Chromatography (HPLC) coupled with Mass Spectrometry (MS), which provides exceptional resolution and specificity for detecting trace levels of impurities . Additionally, advanced methods like Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) are used to quantify these impurities with high precision [15]. The integration of automated sample preparation techniques, such as solid-phase extraction (SPE), further enhances efficiency and accuracy, minimizing contamination and variability. These state-of-the-art approaches are crucial for meeting stringent regulatory guidelines [9] and for ensuring the safe use of pharmaceutical products by detecting harmful impurities at levels that could pose risks to human health.

Analytical Technique Design:
HPLC (High-Performance Liquid Chromatography) analytical technique design is crucial to the precise quantification and analysis of active pharmaceutical ingredients (APIs) and their impurities. The HPLC method development begins by defining objectives, understanding analyte properties (pKa, solubility, polarity), and selecting an appropriate chromatographic mode (RP, NP, IEC, SEC). Mobile phase composition is optimized with buffers (phosphate, acetate) and organic solvents (ACN, MeOH) while adjusting pH. Column selection (C18, C8, Phenyl, CN columns) and detection method (UV, MS, RI, CAD) depend on analyte characteristics. Isocratic or gradient elution is optimized, followed by adjustments to flow rate, temperature, and injection volume. The method is validated for accuracy, precision, linearity, robustness, and system suitability, ensuring reliable, reproducible results for quantitative and qualitative analysis in pharmaceutical and research applications. It aids in verifying the purity, potency, and stability of drug products, ensuring regulatory compliance [16-17]. Step by step HPLC method development defined as below.

Chart-1: Schematic flow diagram representing the step-by-step HPLC method developme
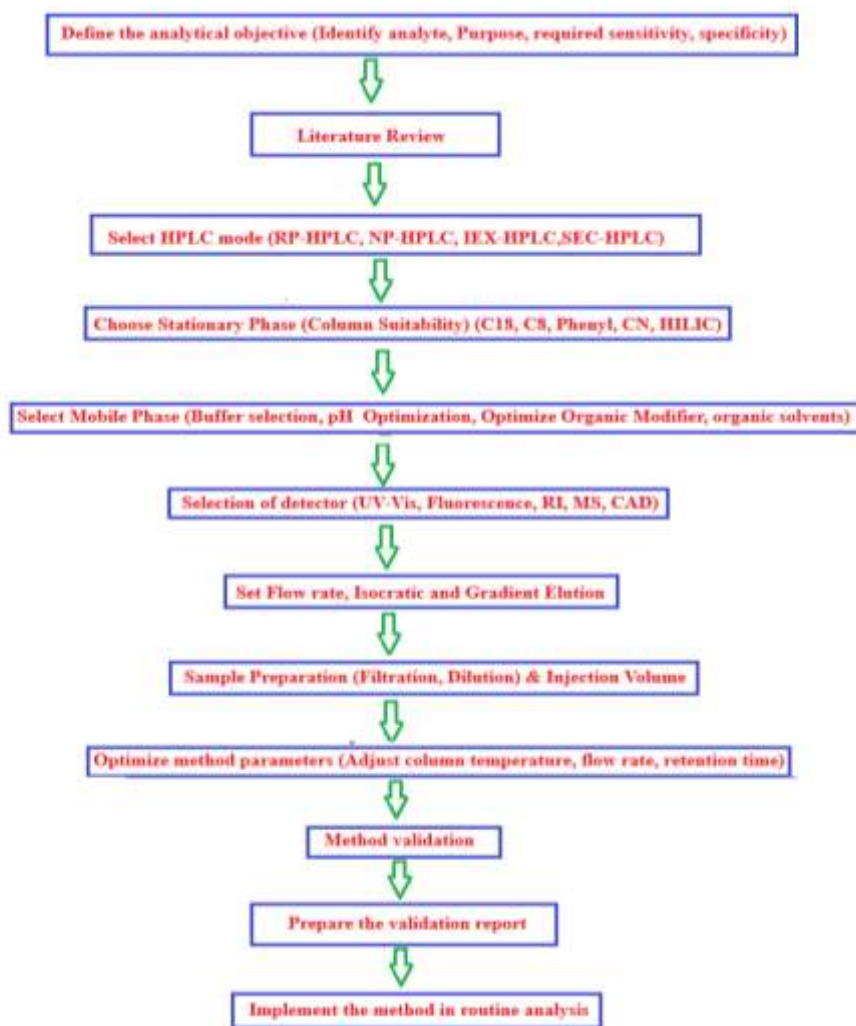
Defining the analytical objective / Formulating the Analytical Objective:
Defining the analytical objective in HPLC method development requires a clear understanding of the target analyte's chemical properties, intended application, and performance criteria such as specificity, sensitivity, and linearity. The objective should guide the selection of key method parameters, including column chemistry, mobile phase composition, gradient profile, and detection mode, to optimize separation efficiency and reproducibility. Consideration of sample matrix effects, potential interferences, and system suitability requirements ensures method robustness and reliability. Additionally, compliance with regulatory guidelines (e.g., ICH Q2(R1)) is essential to validate the method for its intended purpose, ensuring accuracy and precision in quantitative or qualitative analysis.

The selection of the appropriate HPLC mode
The selection of the appropriate HPLC mode in method development is driven by the physicochemical properties of the analyte, including polarity, molecular weight, and ionizability, to achieve the desired separation and detection. Reversed-phase HPLC is commonly used for non-polar to moderately polar compounds, while normal-phase, ion-exchange, or size-exclusion chromatography is chosen for highly polar, ionic, or large biomolecules, respectively. The choice of mode must align with the analytical objective, ensuring optimal resolution, sensitivity, and robustness while minimizing matrix effects and interferences.

Literature Review:-

A thorough literature review in HPLC method development involves critically assessing existing chromatographic techniques, methodologies, and conditions previously applied to similar analytes or matrices. This process identifies key parameters such as mobile phase composition, column selection, and detection modes, helping to establish a baseline for method optimization. It also highlights potential challenges, including issues with resolution, peak tailing, and matrix interference, and provides insight into successful strategies for overcoming these hurdles. By leveraging past studies, the method development process can be streamlined to ensure reliability, reproducibility, and regulatory compliance.

Column Suitability:

Column selection in HPLC is crucial for achieving effective separation, resolution, and sensitivity based on the analytes' characteristics. C18 columns, widely used in reversed-phase chromatography, are versatile for a range of compounds and come in various lengths (e.g., 250 mm for high-resolution separations and 150 mm for faster analysis) and particle sizes (5 µm for standard separations, 3 µm or smaller for higher resolution and faster analysis). Phenyl and cyanopropyl (CN) columns offer selectivity for aromatic and polar compounds, respectively. Phenyl columns interact with aromatic compounds through π-π interactions, while CN columns target polar analytes using dipole-dipole interactions. Silica-based columns are typically used for normal-phase chromatography, providing excellent separation for non-polar and slightly polar compounds, while amino columns are ideal for polar analytes, such as sugars and amino acids. The choice of particle size, stationary phase, and pore size impacts resolution, analysis time, and efficiency. Optimizing column parameters ensures optimal separation and performance across various HPLC applications [3].

Mobile Phase Fine-tuning:

Fine-tuning the mobile phase is essential for optimizing separation efficiency, resolution, and peak shape in HPLC. Adjusting solvent composition, pH, and ionic strength can significantly impact retention times and overall chromatographic performance [18].The nature of the product and molecular structure play a crucial role in mobile phase optimization. Polar compounds require higher aqueous content in reversed-phase chromatography, whereas non-polar compounds benefit from increased organic solvent concentration. pH adjustments are critical for ionizable compounds, as tuning near the pKa improves peak shape and retention. Basic compounds perform well in acidic conditions, while acidic compounds require slightly basic mobile phases. Solvents like methanol or acetonitrile impact selectivity, especially for aromatic compounds. Buffer strength and ionic strength further stabilize retention and improve resolution, ensuring optimal HPLC performance.

Choice of Buffer:

Buffers are essential in HPLC method development, stabilizing pH, improving peak shape, and enhancing reproducibility [6]. Phosphate buffers (pH 2.0–8.0) are widely used due to strong buffering capacity and UV compatibility, while acetate (pH 3.5–5.5) and formate (pH 2.5–4.5) buffers are ideal for MS applications. Tris (pH 7.0–9.0) is suitable for biomolecules, while citrate (pH 3.0–6.2) aids complex separations but may interact with metal surfaces. The product's nature influences buffer selection; acidic compounds need slightly acidic buffers for proper ionization, while basic compounds perform better in acidic mobile phases. Polar analytes require lower organic content, whereas hydrophobic molecules need higher organic content for proper elution. Optimizing buffer selection ensures stability and reproducibility.

pH optimization:

pH optimization is a vital step in HPLC method development, influencing the ionization state of analytes and thereby affecting retention, resolution, and peak shape. Adjusting pH ensures reproducibility and prevents issues such as peak tailing and baseline noise [6]. Acidic compounds perform best at low pH, where they remain in their neutral form for optimal separation. Basic compounds require an acidic mobile phase to reduce ionization and enhance peak sharpness. Neutral compounds need minimal pH adjustments, though ionic strength optimization can improve retention. pH optimization is particularly important for complex mixtures and biological samples, where precise separation and stability are crucial.

Contribution of organic modifiers:

Organic modifiers play a significant role in HPLC method development by altering the polarity of the mobile phase, affecting analyte retention and separation. Modifiers like methanol, acetonitrile, and ethanol reduce the mobile phase's polarity, enhancing the elution of hydrophobic compounds and improving resolution [6]. For non-polar compounds,

higher concentrations of organic modifiers are used, while polar compounds often require lower organic content. The nature of the product influences the choice of organic modifier; for example, hydrophobic compounds require more organic solvent for faster elution, while polar molecules may need a more aqueous-rich phase for optimal separation. Organic modifiers also impact detection sensitivity, particularly in UV and MS detection.

Opting for Detectors in Chromatography:
Choosing the appropriate detector is crucial in HPLC method development, as it directly affects sensitivity, selectivity, and the ability to detect analytes at trace levels. Common detectors include UV-Vis, fluorescence, refractive index (RI), and mass spectrometry (MS). UV detectors, Charged Aerosol Detector CAD detectors, are widely used for compounds with chromophores, while fluorescence detectors offer high sensitivity for specific analytes. The product nature dictates the detector choice; for non-chromophoric or non-fluorescent compounds, MS or RI detectors may be more suitable [19]. For volatile or thermally stable compounds, MS provides excellent sensitivity and structural information, while UV detectors work well for analytes with known absorbance. Optimizing detector selection based on analyte properties ensures accurate quantification and enhanced method performance.

Flow rate optimization:
Flow rate in HPLC is a critical parameter affecting retention time, peak resolution, and column efficiency. An optimal flow rate ensures proper interaction between the analyte and stationary phase, balancing separation quality and analysis time. Excessive flow rates can reduce resolution and increase backpressure, while low flow rates may lead to peak broadening and longer run times. Maintaining an appropriate flow rate is essential for method robustness, reproducibility, and column longevity [6].

Set isocratic and gradient elution:
Isocratic and gradient elution are essential techniques in HPLC for optimizing separation efficiency. Isocratic elution maintains a constant mobile phase composition, suitable for simple mixtures with stable retention times, ensuring method reproducibility. Gradient elution, by varying solvent strength over time, enhances resolution for complex mixtures, reducing analysis time and improving peak shape. Proper selection between these modes ensures optimal analyte separation, sensitivity, and robustness in chromatographic analysis [6-18].

Preparation of Analytical Samples:
Sample preparation is a critical step in HPLC method development, as it directly influences the accuracy, precision, and reproducibility of results. Proper sample preparation ensures that analytes are in a suitable form for analysis, minimizing matrix effects, contamination, and interference. Depending on the product nature, the sample may require filtration, dilution, or extraction [21]. For biological or complex samples, techniques like solid-phase extraction (SPE) or liquid-liquid extraction (LLE) may be employed to isolate the analyte. The sample matrix can also impact chromatographic behavior, so adjusting factors like pH or solvent composition may be necessary for optimal separation. Ensuring sample integrity before injection is key to reliable HPLC results.

Injection volume optimization:
Injection volume in HPLC is a critical parameter influencing chromatographic efficiency, peak shape, and method sensitivity. An optimal injection volume ensures proper analyte dispersion, preventing peak broadening, tailing, or column overloading. It must be compatible with column dimensions, mobile phase composition, and detector sensitivity to maintain resolution and reproducibility. Excessive injection volumes can lead to band broadening, while insufficient volumes may reduce detection sensitivity [6].

Method optimization:
HPLC method optimization involves systematically refining chromatographic conditions to achieve optimal separation, resolution, and sensitivity. It begins with selecting initial conditions based on literature or prior experience. Mobile phase composition is adjusted by varying organic solvent ratios, buffer concentration, and pH. Column selection is optimized by testing different chemistries (C18, C8, HILIC), lengths, and particle sizes. Flow rate, temperature, and gradient/isocratic elution are fine-tuned for better peak resolution. Injection volume and sample solvent compatibility are assessed. Peak shape and system suitability parameters are evaluated, followed by robustness testing. Once optimized, the method undergoes validation before routine application [22].

Table 1: Details of method development various pharmaceutical compounds:

Name of the product / Objective	Chromatographic Conditions	Findings & results	Citation
Name of the product: Cabotegravir. **Objective** :Develop an analytical method for the determination of related substances (RS) and degradation products of Cabotegravir using Quality by Design (QbD) principles.	**Column:** BEH Phenyl (1.7 µm, 150 × 2.1 mm) **Mobile Phase A:** Ammonium formate buffer (pH = 3.5) **Mobile Phase B:** Acetonitrile **Detector:** PDA **Wavelength:**258 nm. **Flow Rate:** 0.36 mL/min **Elution mode:** Gradient:	Successfully separated Cabotegravir and its related substances.	[23]
Name of the product: Ursodeoxycholic Acid. **Objective** : Develop an HPLC-CAD method for the simultaneous quantification of nine related substances in Ursodeoxycholic Acid (UDCA) and identification of two unknown impurities	**Column:**Zorbax Eclipse XDB C18 (250 × 4.6 mm, 5 µm) **Mobile Phase A:** Ammonium formate buffer (pH 3.5) **Mobile Phase B:** Methanol-Acetonitrile (60:40) **Detector:** Charged Aerosol Detector (CAD) **Flow Rate:** 0.8 mL/min **Elution mode:** Gradient	Developed a novel HPLC-CAD method for simultaneous impurity quantification. Achieved improved sensitivity.	[24]
Name of the product: Metformin Hydrochloride & Teneligliptin **Objective:** Develop and validate an RP-HPLC method for impurity profiling in combination tablet dosage form..	**Column:** BDS Hypersil C18 (250 × 4.6 mm, 5 µm) **Mobile Phase:** Octane sulfonic acid & phosphate buffer (pH 3.0) as Mobile Phase A; **Mobile Phase B :** Acetonitrile **Flow Rate:** 1.0 mL/min **Wavelength:** 210 nm **Injection Volume:** 20 µL **Column Temperature:** 35°C **Elution Mode:** Gradient	During the stress study with acid, base, peroxide and temperature, maximum degradation was observed with peroxide indicating the sensitivity of the molecule toward oxidative stress.The developed method was validated	[25]
Name of the product Amlodipine Besylate **Objective:** Develop a stability-indicating HPLC-UV method for Amlodipine Besylateand its impurities.	**Column:** Core shell C18 100mm X 4.6mm, 2.6um **Mobile Phase:-A** 0.4% Ammonium hydroxide, **Mobile-B:** Methanol **Flow Rate:** 1.0mL **Wavelength:** 237 **Column Temperature:** 35 **Elution Mode:** Gradient	The method successfully separated Amlodipine and its impurities. Stability studies confirmed degradation under stress conditions.	[26]
Name of the product: Zuclopenthixol **Objective:** Develop a stability-indicating HPLC method for quantification of pharmacopoeia impurities of Zuclopenthixol and characterization of its stress degradation products by LCMS/MS	**Column:** KNAUER C18 (250 mm × 4.6 mm, 5 µm) **Mobile Phase A:** 0.1 M sodium acetate buffer (pH 4.3) and methanol (20:80, v/v) **Mobile Phase B:** 0.1% formic acid and acetonitrile (75:25, v/v) **Flow Rate:** 0.8 mL/min **Elution Mode:** Isocratic **Detector:** UV Detector **Wavelength:** 257 nm **Additional Analysis:** LC-	Successfully identified and characterized six degradation products (DPs) under stress conditions. - The method was found to be highly sensitive (LOD: 0.009 µg/mL). - Stability-indicating capability demonstrated through forced degradation studies	[27]

MS/MS for degradation product characterization.		

Validation of Analytical Techniques:
In chromatography, method validation involves comprehensive testing to ensure that the analytical procedure consistently produces results within specified limits of accuracy, precision, reliability and robustness. Additionally, it confirms that the method is free from interference and can accurately quantify trace levels of analytes within complex matrices, ensuring the reliability of results across different laboratory environments and over extended periods. Moreover, the validation process also evaluates the method's performance under stress conditions, such as variations in temperature, pH, and mobile phase composition, to assess its robustness. This ensures that the method remains stable and effective even with slight procedural or environmental changes, minimizing the risk of unreliable results in routine analysis. Furthermore, the validation process confirms that the method is capable of detecting analytes at the required sensitivity, meeting regulatory requirements, and demonstrating consistent performance across a range of sample types and conditions. Validation ensure that methods comply with regulatory standards [11-22].
Key components of method validation in chromatography include:

Specificity
Definition: Specificity is the ability of an analytical method to measure the analyte of interest in the presence of other components such as impurities, degradation products, or excipients without interference.
Assessment: Specificity is tested by evaluating the method's ability to distinguish the target analyte from potential interfering substances. A method is considered specific if it accurately measures only the analyte without cross-reacting with other substances.

Precision
Definition: Precision refers to the degree of reproducibility or consistency of results when the same sample is analyzed multiple times under the same conditions. High precision means that repeated measurements under the same conditions yield similar results.
Assessment: Precision can be measured as:
Repeatability: The variation in results when the same sample is analyzed multiple times within a short time frame by the same operator and using the same equipment.
Intermediate precision: The variation observed when different operators or equipment are used under slightly different conditions.
Reproducibility: The precision of the method when performed under different conditions, such as different laboratories.

Sensitivity
Definition: Sensitivity refers to the ability of an analytical method to detect low concentrations of the analyte. It is closely related to the limit of detection (LOD) and limit of quantification (LOQ).
Limit of Detection (LOD): The lowest concentration of analyte that can be reliably detected, though not necessarily quantified.
Limit of Quantification (LOQ): The lowest concentration of analyte that can be quantified with acceptable accuracy and precision.
Assessment: Sensitivity is evaluated by determining the LOD and LOQ through calibration curve analysis or signal-to-noise ratio measurements.

Accuracy:
Definition: Accuracy refers to the closeness of the measured value to the true value or the reference standard. In other words, an accurate method produces results that are close to the actual value of the analyte being measured.
Assessment: It is typically evaluated by comparing the results of the analytical method to a known reference standard or using spiked samples with a known concentration of the analyte.
Linearity
Definition: Linearity refers to the ability of an analytical method to provide results that are directly proportional to the concentration of the analyte over a specified range.
Assessment: Linearity is evaluated by analyzing a series of standard solutions with different concentrations of the analyte. The responses (e.g., peak area, absorbance) are plotted against concentration, and a linear relationship should be observed.
Range
Definition: The range refers to the concentration interval within which the analytical method provides accurate and reliable results. It is defined as the difference between the highest and lowest concentrations of the analyte that can be reliably measured with the method.
Assessment: The range is established by testing the method's performance across different concentrations, from low to high, and ensuring the method remains linear and accurate.
Robustness
Definition: Robustness refers to the ability of an analytical method to remain unaffected by small, deliberate variations in method parameters, such as temperature, pH, or solvent composition. It assesses the method's reliability under varied conditions.
Assessment: To test robustness, key method parameters (such as pH, temperature, mobile phase composition, or detector settings) are varied within small, predefined ranges, and the impact on the results is observed.

Table-2: Comparison of HPLC Method Validation for Impurity Profiling in Pharmaceutical products.

Name of the product	Objective	Validation Focus	Findings & Results	Citation
Esomeprazole	Develop a simple stability-indicating HPLC method for estimation of impurities in Esomeprazole (ESO), ensuring optimal resolution and minimal run time.	The method validation include specificity, linearity, accuracy, precision, robustness, solution stability, and forced degradation studies following ICH guidelines.	The method demonstrated high sensitivity with excellent linearity ($R^2 > 0.9999$), precision (RSD < 2%), and robustness. Forced degradation confirmed stability.	28
Acetylsalicylic Acid	Validate an HPLC method for determining salicylic acid and unknown impurities in acetylsalicylic acid pharmaceutical tablets	The method validation includes linearity, precision (repeatability, reproducibility), accuracy, specificity, robustness, stability, and forced degradation.	The method successfully validated within 0.005–0.40% salicylic acid concentration, showing good linearity, precision, accuracy, and robustness.	[29]
Eluxadoline	Develop and validate an RP-HPLC method for determining eluxadoline and its impurities..	The method validation includes specificity, linearity, precision, LOD/LOQ, and robustness.	Linearity was established for EXDL ($R^2 > 0.997$). The method was robust, precise, and sensitive with an LOD of 0.25 μg/mL.	[30]
Diltiazem hydrochloride	Develop a novel gradient stability-indicating RP-HPLC method for quantitative profiling of known, unknown, and degradant impurities in diltiazem hydrochloride tablets.	The method validation covering specificity, accuracy, linearity, robustness, system suitability, and forced degradation analysis.	Under stress conditions the mass balance was 95-105%. Linearity ($R^2 > 0.99$) was established.	[31]

Vision for the Future.
Emerging era of HPLC and LC-MS/MS in pharmaceuticals holds promising advancements, particularly in drug development, quality control, and regulatory compliance. Enhanced sensitivity and resolution in both technologies will allow for more accurate detection of impurities and better quantification of drug compounds at lower concentrations, including genotoxic impurities such as nitrosamines [14] ensuring higher safety standards [1]. Automation and integration of these techniques will streamline workflows, increase throughput, and reduce operational costs, making pharmaceutical testing more efficient and cost-effective. Moreover, the incorporation of green chemistrypractices, such as eco-friendly solvents and energy-efficient systems, aligns with the growing demand for sustainability in pharmaceutical manufacturing [20]. As both technologies evolve, they will continue to play a critical role in meeting stringent regulatory requirements and supporting the development of personalized medicine. The increased accessibility and affordability of these technologies will democratize their use, benefiting smaller labs and facilitating research and diagnostics across the pharmaceutical industry.

Ultra-High-Performance Liquid Chromatography (UHPLC) Advancements
UHPLC builds upon traditional HPLC by using sub-2 µm particle columns and higher pressures (>1500 bar) to achieve faster and more efficient separations. The reduced particle size enhances resolution, peak capacity, and sensitivity, making it ideal for complex sampleanalysis. Futuredevelopments focus on improving system robustness, reducing column degradation, and integrating microfluidic technology to enhance performance while minimizing solvent consumption [32].

Multidimensional & Hybrid Chromatography:
Multidimensional chromatography, such as 2D-LC, enables the separation of highly complex mixtures by using two different separation mechanisms in a single run. HPLC-MS/MS continues to evolve, providing ultra-sensitive detection for pharmaceuticals, biomolecules, and environmental contaminants. Hybrid techniques like HPLC-NMR and HPLC-IR allow simultaneous separation and structural elucidation, facilitating in-depth characterization of unknown compounds in drug development and metabolomics [33].

Miniaturization & Microfluidics:
Micro-HPLC and nano-HPLC systems are being developed to handle minimal sample volumes while maintaining high resolution and sensitivity. Capillary and chip-based chromatography enable faster analysis with reduced solvent consumption, making them suitable for point-of-care testing and real-time environmental monitoring. These advancements aim to create portable, automated systems that can deliver high-performance separation without the need for large-scale instrumentation [34].

AI-driven Technology & Automation:
AI is transforming HPLC by automating method development, optimizing chromatographic conditions, and improving data analysis. Machine learning algorithms help select the best column, mobile phase, and gradient conditions, reducing the need for trial-and-error experimentation. Automated peak detection, deconvolution, and impurity profiling improve reproducibility and reduce human error, making HPLC more efficient in pharmaceutical quality control and research [35].

Green & Sustainable HPLC:
Sustainability in HPLC focuses on reducing solvent waste, using eco-friendly alternatives, and adopting energy-efficient systems. Supercritical Fluid Chromatography (SFC) uses CO_2 as a mobile phase, significantly lowering organic solvent consumption while maintaining high separation efficiency. Additionally, aqueous normal-phase chromatography (ANPC) and biodegradable solvent systems are gaining traction as greener alternatives to traditional reversed-phase chromatography [36].

Advances in Stationary Phases:
The development of core-shell particles, monolithic columns, and molecularly imprinted polymers (MIPs) is improving separation efficiency and selectivity. Core-shell particles reduce backpressure while maintaining high resolution, allowing faster analysis with lower system strain. Monolithic columns offer higher permeability and reduced column resistance, making them ideal for high-throughput applications. MIPs provide tailor-made selectivity for specific analytes, enhancing targeted analysis in pharmaceutical and environmental research [37].

Improved Detection Technologies
Next-generation detectors, such as high-resolution UV-Vis, photodiode array (PDA), and fluorescence detectors, are enhancing sensitivity and dynamic range in HPLC. Advanced electrochemical detectors improve selectivity, especially for biomolecule analysis. The integration of real-time spectroscopic methods, such as inline mass spectrometry or Raman spectroscopy, allows continuous monitoring of chemical processes, reducing analysis time and increasing data reliability [38].

Continuous and High-Throughput Processing:
New developments in sample injection and parallel HPLC systems aim to enhance sample throughput while maintaining precision. Simultaneous Multi-Sample Injection (SMI) techniques reduce downtime by enabling multiple samples to be analyzed in parallel, increasing productivity in pharmaceutical and environmental testing. Continuous HPLCprocessing is also beingexplored to streamline large-scale industrial applications, such as biopharmaceutical production and food safety testing [39].

Integration of HPLC with Emerging Omics Technologies:
HPLC is increasingly being integrated with omics technologies such as proteomics, metabolomics, and lipidomics to analyze complex biological samples with high specificity. Advanced separation techniques coupled with high-resolution mass spectrometry (HPLC-MS) enable precise identification of biomolecules at ultra-trace levels. These integrations are crucial for drug discovery, personalized medicine, and disease biomarker identification, driving innovations in high-throughput bioanalytical workflows [40].

CONCLUSIONS:
High-Performance Liquid Chromatography (HPLC) remains a critical technique in the detection and quantification of process impurities in pharmaceutical compounds. A holistic approach to HPLC, encompassing method development, optimization, and validation, ensures precise, reliable, and reproducible results. Key factors, including analyte properties, column selection, mobile phase composition, and sample preparation, must be carefully optimized to achieve optimal separation and sensitivity. The integration of advanced techniques like UHPLC, mass spectrometry, and AI-driven method optimization enhances HPLC's capability to detect trace-level impurities and improve efficiency. Validation ensures compliance with regulatory standards, ensuring pharmaceutical safety. As technological advancements continue, including miniaturization, hybrid chromatographic methods, and greener practices, HPLC's role in pharmaceutical analysis will expand, offering improved performance, sustainability, and applicability across diverse sectors. Ultimately, HPLC will remain central to ensuring the purity, safety, and efficacy of pharmaceutical products.

List of Abbreviations
HPLC - High-Performance Liquid Chromatography
GTIs - Genotoxic Impurities
UHPLC - Ultra-High-Performance Liquid Chromatography
MS - Mass Spectrometry
AI - Artificial Intelligence
SPE - Solid-Phase Extraction
LLE - Liquid-Liquid Extraction
SFC - Supercritical Fluid Chromatography
SPE - Solid-Phase Extraction
S/N - Signal-to-Noise
LOD - Limit of Detection
LOQ - Limit of Quantification
PDA - Photodiode Array
HPLC-MS/MS - High-Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
MIPs - Molecularly Imprinted Polymers
ANPC - Aqueous Normal-Phase Chromatography
LC-MS/MS - Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
2D-LC - Two-Dimensional Liquid Chromatography

Declarations
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P. Chandrashekhar Reddycollected and analysed the data and wrote the manuscript. He is the lead author and showed strong commitment to the work. Dr. G Sampath Kumar Reddy has made critical suggestions to the conception and substantively revised the work. Dr B. Jainendra Kumarwas the supporting pillar for writing manuscript and reviewed the work. All authors have read and approved the manuscript.

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The authorsproclaim no conflicts interests.

Authors' details:
1. Department of Chemistry, Anurag University, Hyderabad, India.
2. Department of Analytical Research and Development, Aurore Life Sciences Private Limited, Hyderabad, India

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