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Hyphenated techniques in impurity profiling of Efavirenz: A review of LCMS, NMR and HPLC application

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ABSTRACT
The management and assessment of impurities in pharmaceutical products are essential for guaranteeing drug quality and safety. Although impurities may exist in minimal concentrations, they can profoundly impact drug efficacy and lead to adverse effects, including toxicity and carcinogenicity. Impurity profiling encompasses the identification, characterization, and quantification of impurities throughout the drug development process. Advanced analytical techniques, such as liquid chromatography and mass spectrometry, play a crucial role in precise impurity detection and measurement. Regulatory guidelines establish a framework for identifying and managing impurities, thereby enhancing drug safety. Techniques like crystallization and chromatography are key in purifying products and eliminating mutagenic impurities. Understanding the origins and pathways of impurities is crucial for developing effective control strategies. Continuous research and development in advanced analytical methods and regulatory compliance are vital to ensuring the safety and efficacy of pharmaceutical products.

INTRODUCTION:

ABBREVIATION:
HIV -human immunodeficiency virus
DNA- Deoxyribonucleic acid
RNA -Ribonucleic acid
CYP2B6 - Cytochrome P450 2B6
LC-MS- Liquid Chromatography-Mass Spectrometry
ICH -International Council for Harmonisation
^{1}H NMR- proton nuclear magnetic resonance
HPLC- High-Performance Liquid Chromatography
LCMS Liquid Chromatography Mass Spectrometry.
COSY- Correlated Spectroscopy, HSQC- Heteronuclear Single Quantum Coherence
HMBC- Heteronuclear Multiple Bond Correlation

Importance of impurity profiling: The rigorous control and identification of impurities within pharmaceutical products has emerged as a critical element in ensuring both the quality and safety of medications. Pharmaceutical impurities, defined as any unwanted chemical entities present within a drug formulation that are not the active pharmaceutical ingredient or excipients, can arise from various sources, including the synthetic process, degradation during storage, or interactions with formulation components.[1] Even trace amounts of these impurities can exert a significant influence on the efficacy and safety profile of a drug, potentially leading to adverse effects such as toxicity, carcinogenicity, or teratogenicity. Consequently, pharmaceutical companies, regulatory bodies, and patient advocacy groups

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recognize the critical importance of impurity profiling in safeguarding public health.[2,3] The process of impurity profiling involves the identification, characterization, and quantification of impurities present in pharmaceutical materials, encompassing raw materials, intermediates, and finished drug products[1]. Impurities may arise during the synthesis of the active pharmaceutical ingredient from raw materials, intermediates, or by-products due to concurrent reactions, and even seemingly innocuous processes like salt formation can introduce new impurities. The International Council for Harmonisation guidelines play a pivotal role in standardizing the approach to impurity control, facilitating the submission of comprehensive data to regulatory agencies.[2] Impurity profiling is not merely an exercise in analytical chemistry but an essential component of risk assessment and quality control throughout the drug development and manufacturing lifecycle.[4]

The multifaceted nature of impurity profiling necessitates a comprehensive approach that integrates advanced analytical techniques with a deep understanding of chemical processes and regulatory requirements. Modern analytical techniques play a crucial role in identifying and quantifying these impurities, as any pharmaceutical product needs to be free from impurities to serve its intended therapeutic activity.[5] Liquid chromatography coupled with mass spectrometry is frequently employed to detect, identify, and quantify impurities.[6] Impurity control is vital as these impurities can have negative impacts on a drug's efficacy and safety, so strict regulations and guidelines have been put in place to ensure pharmaceutical products are safe and effective for patients. Impurity profiling is crucial for ensuring the quality and safety of pharmaceutical products.[1,7] The process of impurity profiling begins with a thorough understanding of the synthetic route and potential degradation pathways of the drug substance. This knowledge allows for the prediction of possible impurities and the selection of appropriate analytical methods for their detection.[8] The structural identification of an impurity is the initial step in understanding its formation chemistry and subsequent control.[6] When dealing with impurities that have no identified structure, it's helpful to use a decision tree from the ICH to figure out how to handle them properly.[9] Once identified, impurities are characterized in terms of their chemical structure, physicochemical properties, and potential toxicity.

The emphasis shifts from resolution to speed, ruggedness, and robustness in the later stages of the drug development process when the manufacturing processes are being qualified[10]. Impurity characterization extends beyond mere identification and quantification; it also encompasses the assessment of their potential impact on product quality and patient safety. Toxicological studies are conducted to evaluate the potential adverse effects of impurities, and acceptable limits are established based on safety thresholds. If an impurity exceeds the established threshold, steps must be taken to reduce its levels through process optimization or purification techniques. Crystallization is a common unit operation to control mutagenic impurities, relying on impurity purge mechanisms to reject impurities into the liquid phase.[11] The importance of employing orthogonal analytical techniques cannot be overstated, as different methods offer complementary information and can enhance the robustness of impurity profiling studies. Impurities can come from different sources, so knowing where they come from is essential for controlling them effectively.

The implications of impurity profiling extend beyond regulatory compliance, influencing various aspects of pharmaceutical development, manufacturing, and quality control. Early phase material for toxicology studies is often prepared from the same batch as the material used for Phase 1 clinical investigations.[12] By identifying and controlling impurities early in the drug development process, companies can avoid costly delays and potential safety issues later on.

Efavirenz overview: Efavirenz, a non-nucleoside reverse transcriptase inhibitor, has become a cornerstone in the management of human immunodeficiency virus type 1 infection, playing a pivotal role in highly active antiretroviral therapy regimens globally.[13] As a critical component of HIV treatment, efavirenz directly inhibits the reverse transcriptase enzyme, essential for viral replication, thereby reducing the viral load in infected individuals and improving their overall immune function.[14] The widespread use of efavirenz is underpinned by its efficacy, relatively convenient once-daily dosing, and affordable cost, particularly in resource-limited settings where access to newer antiretroviral agents may be restricted.[15] Most patients were initiated on a regimen that included efavirenz, tenofovir disoproxil fumarate, and emtricitabine.[16] However, like all antiretroviral medications, efavirenz is associated with certain challenges, including potential adverse effects and the development of drug resistance, which must be carefully considered in clinical decision-making to optimize patient outcomes.[17] The success of combination antiretroviral therapy has changed the focus to antiretroviral drug regimens that optimize tolerability, long-term safety, and durable efficacy.[18]

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Understanding the intricate pharmacological properties of efavirenz is paramount for healthcare professionals involved in HIV care, necessitating a comprehensive grasp of its mechanism of action, pharmacokinetics, potential drug interactions, and strategies for managing associated toxicities. Efavirenz's mechanism of action centers on its ability to selectively bind to HIV-1 reverse transcriptase, a critical enzyme responsible for converting viral RNA into DNA, thereby disrupting the viral replication cycle.[19] This non-competitive inhibition prevents the synthesis of new viral DNA, leading to a reduction in viral load and subsequent immune reconstitution in treated individuals. The pharmacokinetic profile of efavirenz is characterized by its high oral bioavailability, extensive distribution throughout the body, and metabolism primarily via the cytochrome P450 enzyme system, particularly CYP2B6. Significant interpatient variability in efavirenz plasma concentrations has been observed, suggesting that therapeutic drug monitoring may be advisable.[20]

The clinical efficacy of efavirenz has been extensively demonstrated in numerous clinical trials, establishing its role as a potent antiretroviral agent capable of achieving and maintaining viral suppression in a majority of patients.[16]

However, efavirenz is not without its limitations, as it is associated with a range of adverse effects, including central nervous system toxicities such as dizziness, insomnia, and psychiatric symptoms, which can impact adherence and quality of life.[21] Furthermore, efavirenz has been linked to metabolic complications such as dyslipidemia and lipodystrophy, necessitating careful monitoring and management to mitigate long-term health risks.

Resistance to efavirenz can develop through mutations in the reverse transcriptase gene, particularly the K103N mutation, which is commonly associated with virological failure.

Strategies to minimize resistance include adherence counseling, selection of alternative antiretroviral agents in cases of intolerance or contraindications, and consideration of drug resistance testing to guide treatment decisions.

The role of efavirenz in HIV treatment continues to evolve as newer antiretroviral agents with improved safety profiles and enhanced efficacy become available.

IMPURITY PROFILING TECHNIQUES:

LCMS (Liquid Chromatography-Mass Spectrometry): The imperative to identify and control impurities in pharmaceutical products stems from their potential to induce adverse effects, thereby compromising both the quality and safety of the medication.[6] Impurities, defined as any unwanted chemicals that remain within a formulation, necessitate stringent control due to their potential carcinogenic, toxic, or teratogenic effects.[1] Consequently, regulatory bodies mandate the identification and quantification of impurities exceeding specified thresholds.[1,6] Impurity profiling, which meticulously describes both identified and unidentified impurities present in drug products, has gained prominence in pharmacopeias, with the International Council for Harmonisation issuing guidelines for analyzing impurities in new drug substances.[2] Liquid chromatography-mass spectrometry stands out as a pivotal analytical technique for characterizing minor components, including impurities and degradation products, in drug substances and products.[22]

Liquid chromatography, particularly when coupled with mass spectrometry, has revolutionized the detection and identification of impurities in pharmaceutical compounds like Efavirenz.[23] The versatility of LC-MS enables the separation of complex mixtures based on their physicochemical properties, followed by mass spectrometric analysis that provides structural information, facilitating the identification of even trace amounts of impurities.[24]

High-performance liquid chromatography, when coupled with mass spectrometry fulfills the stringent requirements for quality control, offering a wide array of technical options and applications.[25] The advent of ultra-performance liquid chromatography has spurred a revolution in liquid chromatography, bringing regular analysis of new levels of efficiency.[26] With enhanced resolution, sensitivity and speed, this technique is well suited for the analysis of complex samples and can be used with mass spectrometry to estimate food contaminants.[27] Different analytical techniques are used to determine impurities in pharmaceuticals.[7] Spectroscopic methods, chromatographic techniques, and electrochemical methods are a few of them. Among chromatographic techniques, gas chromatography and high-performance liquid chromatography are the most common ones.

Liquid chromatography-mass spectrometry offers several advantages in impurity analysis, including high sensitivity, selectivity, and the ability to analyze a wide range of compounds without derivatization.[28] LC-MS's capacity to integrate separation and detection makes it invaluable for impurity analysis, especially in complex matrices.[29] Furthermore, advances in mass spectrometry, such as high-resolution mass spectrometry, provide

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accurate mass measurements that aid in the structural elucidation of unknown impurities. The advances in electronics and computer-assisted data processing have provided powerful instruments that obtain more information than can be analyzed by using traditional data analysis methods.[30] The technology has been used to determine the presence of contaminants in food products.[31]
The application of LC-MS in detecting and identifying impurities in Efavirenz involves several key steps, starting with sample preparation to extract and concentrate the analytes of interest, followed by chromatographic separation to resolve the components of the mixture, and finally, mass spectrometric detection to identify and quantify the impurities present. The sensitivity of LC-MS allows for the detection of impurities at very low concentrations, often below the levels achievable by other analytical techniques.[32] The mobile phase is moved either by a pump.[33] The HPLC method applies to several categories of substances: carbohydrates, lipids, vitamins, additives, synthetic colorings, natural pigments, contaminants, and amino acids.[34]

The robustness and reliability of LC-MS methods are crucial for ensuring the quality and safety of Efavirenz, requiring careful method validation to demonstrate specificity, linearity, accuracy, precision, and sensitivity. The determination of solubility is done by weighing the compound and placing it in test tubes with solvent, shaking until the solute dissolves with vigorous agitation. Moreover, the utilization of internal standards can further improve accuracy and precision.[35] The use of appropriate clean-up techniques, such as solid phase extraction, can significantly reduce matrix interferences and improve signal to noise, enabling mass spectral confirmation and a decrease in the limits of quantification.[36] The matrix, co-extracted with the analytes can alter the signal response, causing either suppression or enhancement, resulting in poor analytical accuracy, linearity, and reproducibility.[37]

^{1}H NMR (Proton Nuclear Magnetic Resonance): Proton Nuclear Magnetic Resonance (1H NMR) spectroscopy stands as a cornerstone analytical technique in the realm of chemistry, offering unparalleled insights into the structure and dynamics of molecules, and playing a pivotal role in the identification and characterization of impurities within complex chemical mixtures.[38,39] The technique hinges on the fundamental principle that atomic nuclei with an odd number of protons or neutrons possess a magnetic moment, rendering them responsive to an externally applied magnetic field.[40] When a sample is placed in a magnetic field, these nuclei align either with or against the field, creating distinct energy levels.[41] Upon irradiation with radiofrequency energy, transitions between these energy levels occur, generating a spectrum that provides a wealth of information about the chemical environment of each proton within the molecule.[42]

The power of 1H NMR spectroscopy in impurity analysis stems from its ability to resolve individual signals corresponding to different protons within a molecule, with the position of each signal, known as the chemical shift, being exquisitely sensitive to the electronic environment surrounding the proton.[43] Electronegative atoms or groups in close proximity to a proton deshield it, causing its signal to shift downfield to higher chemical shift values, while electron-donating groups shield the proton, resulting in an upfield shift . Furthermore, the integration of each signal is directly proportional to the number of protons it represents, providing quantitative information about the relative abundance of different components in a mixture. The multiplicity of a signal, arising from spin-spin coupling between neighboring protons, further elucidates the connectivity and spatial arrangement of atoms within the molecule. By meticulously analyzing the chemical shifts, integrations, and multiplicities of signals in a 1H NMR spectrum, chemists can piece together the structural puzzle of unknown impurities, even when present in trace amounts.[44]

The application of 1H NMR spectroscopy to impurity analysis is particularly valuable in various fields, including pharmaceutical chemistry, environmental science, and materials science. In the pharmaceutical industry, the presence of even trace amounts of impurities in drug substances or products can have a significant impact on their safety and efficacy, making their identification and quantification paramount.[45] 1H NMR spectroscopy provides a powerful tool for detecting and characterizing these impurities, ensuring the quality and integrity of pharmaceutical products. In environmental science, 1H NMR spectroscopy can be used to identify and quantify pollutants in water, soil, and air samples. In materials science, 1H NMR spectroscopy can be employed to characterize the composition and structure of polymers, ceramics, and other materials, as well as to identify any impurities that may be present.

Beyond the standard 1H NMR experiment, a variety of advanced NMR techniques can be employed to further enhance the structural elucidation of impurities. Two-dimensional NMR techniques, such as COSY, HSQC, and HMBC, provide valuable information about the connectivity between protons and other nuclei, such as carbon, enabling the unambiguous assignment of signals and the determination of complex molecular structures.[46]

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Quantitative NMR techniques, such as qHNMR, allow for the accurate determination of the concentrations of different components in a mixture, providing crucial information for impurity profiling and quantification. Furthermore, techniques like NMR-based metabolomics are ideal choices for the comparison and identification of chemical differences.[47] These advanced NMR techniques, coupled with the power of computational chemistry and spectral databases, provide a comprehensive toolkit for tackling even the most challenging impurity analysis problems.[48] Low-field NMR spectrometers present a cost-effective and portable alternative for forensic drug analysis, especially when traditional methods fail to differentiate regioisomers caused by functional group shifts on aromatic rings.[49]

The non-destructive nature of NMR spectroscopy makes it a particularly attractive technique for impurity analysis, as it allows for the recovery of the sample after analysis, enabling further characterization using other analytical techniques. Moreover, the minimal sample preparation requirements of NMR spectroscopy make it a rapid and efficient technique, allowing for high-throughput analysis of multiple samples.[50] While traditional separation techniques like thin-layer chromatography, liquid chromatography, gas chromatography, and capillary electrophoresis coupled with mass spectrometry are used for identifying, quantifying, and structurally elucidating compounds from plant matrices and other sources, NMR offers a complementary approach that can provide unique insights into the structure and dynamics of impurities.[51] By integrating NMR data with information obtained from other analytical techniques, such as mass spectrometry.[52], a more complete and accurate picture of the impurity profile can be obtained. Structure elucidation parameters, such as accurate mass, MS/MS fragmentation patterns, and NMR spectra, can be combined into a single cheminformatics analysis platform to accurately identify unknown metabolites in untargeted studies.[53] The specificity of mass spectrometry is suited for examining complex molecules present in agriculture, atmospheric chemistry, biomedicine, food, forensics, and geochemistry.[54] Chemometrics can be used with mass spectrometry, chromatographic, electrochemical, and thermal methods to analyze data.[30] The ongoing development of new NMR hardware and software continues to push the boundaries of what is possible in impurity analysis.

HPLC (High-Performance Liquid Chromatography): High-Performance Liquid Chromatography stands as a pivotal analytical technique, widely employed for its ability to separate, identify, and quantify individual components within complex mixtures.[34] HPLC's versatility stems from its applicability to a broad spectrum of compounds, encompassing pharmaceuticals, natural products, polymers, and various chemical entities, provided they exhibit solubility in a suitable liquid mobile phase.[55] The fundamental principle underpinning HPLC involves the partitioning of analytes between a stationary phase, typically a solid material packed within a column, and a liquid mobile phase that carries the sample through the column.[56] The differential affinity of analytes for the stationary and mobile phases leads to their separation, as compounds with a stronger affinity for the stationary phase elute later than those with a greater affinity for the mobile phase.[57] The technique's widespread adoption is further propelled by its adaptability to diverse detection methods, including UV-visible absorption, fluorescence, mass spectrometry, and refractive index detection, allowing for the sensitive and selective detection of a wide array of analytes.[32]

The ongoing evolution of HPLC encompasses advancements in column technology, instrumentation, and data processing, continually enhancing its performance and expanding its application scope. Modern trends in HPLC are characterized by the utilization of sub-2 μm particle size packed columns, facilitating ultra-high-pressure liquid chromatography separations and porous-shell particle packed columns to achieve high-efficiency separations with reduced column back-pressures.[29] These innovative column technologies translate to improved resolution, enhanced sensitivity, and accelerated analysis times, catering to the increasing demands for high-throughput analysis in diverse fields.[26] Furthermore, the integration of HPLC with mass spectrometry has revolutionized analytical capabilities, enabling the structural elucidation and quantitation of trace-level analytes in complex matrices.

The advances in separation speed have been mostly related to the development of column technology and instrumentation.[58] One of the most popular techniques to decrease analysis time and increase resolution is using columns packed with sub-2 μm particles to facilitate ultra-high-pressure liquid chromatography separations.[59]

Sample preparation constitutes a critical step in HPLC analysis, often determining the accuracy and reliability of the obtained results. Efficient sample preparation techniques are indispensable to mitigate matrix effects, preconcentrate analytes, and remove interfering substances that could compromise separation or detection.[60] Common sample preparation methods include liquid-liquid

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extraction, solid-phase extraction, and protein precipitation, each tailored to specific sample types and analyte characteristics.[61] Moreover, the rise of miniaturized and automated sample preparation platforms has significantly streamlined workflows, reducing manual handling, minimizing solvent consumption, and enhancing reproducibility.

The choice of stationary phase is paramount in HPLC, dictating the selectivity and efficiency of the separation process. Reversed-phase chromatography, employing nonpolar stationary phases such as C18 or C8 bonded silica, is the most prevalent mode, particularly well-suited for separating nonpolar and moderately polar compounds. Normal-phase chromatography, utilizing polar stationary phases like silica or amino-bonded silica, finds utility in separating polar compounds. Ion-exchange chromatography separates molecules based on their charge, making it suitable for the analysis of ionic species like proteins and amino acids, while size-exclusion chromatography separates molecules based on their size, useful for characterizing polymers and biomolecules.[52] The optimization of mobile phase composition, including pH, solvent strength, and the addition of modifiers, further refines separation selectivity and peak shape.

The applications of HPLC span a vast array of scientific disciplines, including pharmaceutical analysis, environmental monitoring, food chemistry, clinical diagnostics, and proteomics. In the pharmaceutical industry, HPLC plays a crucial role in drug development, quality control, and pharmacokinetic studies, ensuring the safety and efficacy of pharmaceutical products[62]. Its capability to purify amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, antibiotics, and steroids is invaluable.[63] In environmental monitoring, HPLC is employed for the determination of pollutants, pesticides, and herbicides in water, soil, and air samples, safeguarding environmental quality. The versatility of HPLC extends to clinical diagnostics, where it aids in the identification and quantification of biomarkers, therapeutic drug monitoring, and the diagnosis of metabolic disorders.[64] HPLC serves as a powerful tool for the isolation and purification of target compounds from complex mixtures.[65] In the realm of downstream process development, hydrophobic interaction chromatography has emerged as a valuable technique, offering an orthogonal approach to conventional chromatography principles.[66] Chromatography is used as a method of quantitative analysis apart from its separation, which is used to achieve a satisfactory separation within a suitable time interval.[63] The technique boasts high selectivity, separation efficiency and resolution.[33] Different components of mixtures stay longer in the stationary phase because of their differences, and they move slowly in the chromatography system, while others pass rapidly into the mobile phase, and leave the system faster.[63]

IMPURITY PROFILING OF EFAVIRENZ:

Types of impurities: Efavirenz, a non-nucleoside reverse transcriptase inhibitor, serves as a crucial component in the treatment of human immunodeficiency virus type 1 infection. Its efficacy in reducing viral load and improving patient outcomes has made it a cornerstone of antiretroviral therapy.[13,14] However, the presence of impurities in efavirenz drug products raises concerns about safety, efficacy, and overall quality, necessitating a thorough investigation into their sources and potential impact. Impurities in pharmaceutical products can diminish the therapeutic benefits and may lead to harmful effects, thus quality and safety considerations make their control or limitation necessary. Impurities in efavirenz can arise from various sources, including the synthetic process, degradation pathways, and contamination during manufacturing or storage.[6] Identification and control of these impurities are crucial to ensure the drug's safety and efficacy.[67]

Synthesis-related impurities may arise from incomplete reactions, side reactions, or the use of specific reagents and catalysts during the manufacturing process. The starting materials, intermediates, and by-products involved in the synthesis can all contribute to the impurity profile of the final drug substance. Raw materials are often produced to lower quality standards than the final drug, which explains why they might have impurities that eventually compromise the purity of the finished product[2]. It is imperative to optimize the synthetic route to minimize the formation of these impurities through meticulous control of reaction conditions, purification techniques, and the quality of raw materials. Additionally, proper analytical methods are needed to detect and quantify these impurities in accordance with regulatory requirements. Understanding the synthetic pathway and potential side reactions is crucial for identifying and controlling these impurities. In the synthesis of ezetimibe, process-related impurities were detected using HPLC analysis.[68] The International Council for Harmonisation defines an impurity as any component that is not the active pharmaceutical ingredient or an excipient in the drug formulation. It is essential to consider that these unwanted chemicals may have toxic, carcinogenic, or teratogenic effects, which makes it essential to identify, isolate, and characterize them as part of pharmaceutical development. Degradation products are another significant class of impurities that can

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form during the storage and handling of efavirenz. These impurities arise from chemical reactions such as oxidation, hydrolysis, photolysis, or thermal degradation, influenced by factors like temperature, light, pH, and humidity.[1] Efavirenz, like many organic molecules, is susceptible to degradation over time, leading to the formation of new chemical entities that may compromise the drug's quality and safety.[69] Stress testing, a process that involves exposing the drug substance to various environmental conditions, helps identify potential degradation pathways and products.[2] Understanding these pathways is essential for developing appropriate storage conditions, packaging, and formulations that minimize degradation. Forced degradation studies are usually used to determine the chemical stability, pathways of degradation, to identify the degradation products, conditions of storage, self-life, excipient compatibility, and also allow the development.[70] and validation of stability indicating analytical procedures.[71] Some software and databases can predict how a pharmaceutically active substance will react when exposed to deterioration, which can be helpful in determining the primary degradation routes and the primary degradation products that form during pharmaceutical product storage.[69]

Contaminants represent another category of impurities that can be introduced during the manufacturing process or through environmental exposure. These can include inorganic salts, heavy metals, residual solvents, particulate matter, or microbial contaminants. Ensuring the quality of water used in pharmaceutical manufacturing is critical, as water can be a major source of contamination. Microbial contamination is especially problematic in non-sterile products, where microorganisms can proliferate and potentially cause harm to patients.[72] Cleaning procedures are essential to prevent cross-contamination between different products manufactured in the same facility.[73] To prevent the formation of impurities in pharmaceutical products, manufacturers must adhere to strict quality control measures and good manufacturing practices.

Stringent controls on raw materials, manufacturing processes, and storage conditions are necessary to minimize the risk of contamination and ensure the purity of efavirenz drug products. Impurities may come from raw materials, manufacturing supplies, packing materials, or the production process itself.[72] The limit and nature of these contaminants should be carefully controlled to guarantee patient safety and product efficacy.[72,74] Effective cleaning procedures are essential to get rid of pollutants left over from earlier products, residues from cleaning agents, and to manage any microbial pollutants.[73] To maintain drug product safety and efficacy, pharmaceutical companies should thoroughly investigate and control impurities in Efavirenz, protecting public health and guaranteeing the availability of high-quality medications.

LCMS analysis Overview of LCMS methods for detecting and identifying impurities in Efavirenz. Liquid Chromatography-Mass Spectrometry has emerged as an indispensable analytical technique for the detection and identification of impurities in pharmaceutical compounds, offering high sensitivity, selectivity, and the capability for structural elucidation. Efavirenz, a non-nucleoside reverse transcriptase inhibitor widely used in the treatment of human immunodeficiency virus infection, is subject to the formation of impurities during its synthesis, formulation, and storage, necessitating robust analytical methods for their detection and control. Impurities in pharmaceutical products, even in trace amounts, can significantly impact drug safety and efficacy, making their identification and quantification crucial for ensuring patient well-being. Understanding the chemistry of the impurity formation and controlling the level of it is important for the quality and safety considerations.[6] LCMS provides valuable insights into the nature and origin of these impurities, aiding in the optimization of manufacturing processes and the development of more stable and efficacious drug formulations.[24]

The application of LCMS in the analysis of Efavirenz impurities involves several key steps, beginning with sample preparation techniques designed to extract and concentrate the target analytes from the drug substance or product. Various extraction methods, such as liquid-liquid extraction and solid-phase extraction, can be employed to selectively isolate Efavirenz and its related impurities from the sample matrix.[35] The choice of extraction method depends on the physicochemical properties of the target analytes and the nature of the sample matrix. Following sample preparation, chromatographic separation is performed using high-performance liquid chromatography to resolve the individual components of the sample based on their differential interactions with the stationary and mobile phases.[67] Reversed-phase chromatography is commonly used for the separation of Efavirenz and its impurities, employing a hydrophobic stationary phase and a polar mobile phase gradient to achieve optimal resolution. Optimization of chromatographic conditions, including column type, mobile phase composition, flow rate, and temperature, is critical for achieving baseline separation of the target analytes.

Mass spectrometry detection is then employed to

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identify and quantify the separated compounds based on their mass-to-charge ratios. Electrospray ionization and atmospheric pressure chemical ionization are the most common ionization techniques used in LCMS analysis of pharmaceuticals, allowing for the efficient ionization of Efavirenz and its impurities. The mass analyzer separates ions based on their mass-to-charge ratios, generating mass spectra that provide valuable information about the molecular weights and structures of the compounds. Tandem mass spectrometry, also known as MS/MS, is often employed to enhance selectivity and sensitivity by fragmenting the precursor ions and analyzing the resulting fragment ions.[75] The mass fragmentation pattern can be obtained to determine the molecular weight of the compounds. High-resolution mass spectrometry, such as time-of-flight mass spectrometry, provides accurate mass measurements that can be used to determine the elemental composition of the compounds.[28]

Liquid chromatography combined with mass spectrometry and tandem mass spectrometry has become a powerful tool for the identification and characterization of psychoactive substances as well as degradants, metabolites, and process impurities.[23]

Data analysis involves the processing of mass spectral data to identify and quantify the detected impurities. By hyphenating high performance liquid chromatography and mass spectrometry these high demands are fulfilled, providing the user with a multitude of technical options and applications.[25] The acquired data is compared with reference standards or library data to confirm the identity of known impurities. Quantitative analysis is performed by constructing calibration curves using authentic standards of the target impurities. The concentration of impurities in the sample is determined by comparing the peak areas or peak heights of the impurities to those of the standards. Software solutions and prediction systems also aid in the identification and structure elucidation of unknown compounds.[76]

^{1}H NMR Analysis for Structural Elucidation of Impurities in Efavirenz: The identification and characterization of impurities in pharmaceutical products are crucial steps in ensuring drug safety and efficacy, as these unintended components can have adverse effects and must be carefully controlled. High-resolution analytical techniques play a pivotal role in this process, with nuclear magnetic resonance spectroscopy standing out as a powerful tool for structural elucidation.[6] Specifically, proton nuclear magnetic resonance (1H NMR) spectroscopy provides detailed information about the number, type, and connectivity of hydrogen atoms within a molecule, enabling the determination of the structure of unknown impurities present in a drug substance like efavirenz. The analysis of 1H NMR spectra involves several key parameters, including chemical shifts, signal intensities, splitting patterns (multiplicity), and coupling constants, each of which provides unique insights into the molecular environment of the protons being observed.[77] Chemical shifts, measured in parts per million (ppm), are highly sensitive to the electronic environment surrounding each proton, with electronegative atoms or groups causing downfield shifts (higher ppm values) and electron-donating groups causing upfield shifts . Signal intensities are directly proportional to the number of protons contributing to each signal, facilitating the quantification of different structural components within the molecule. Signal splitting, or multiplicity, arises from spin-spin coupling between neighboring protons, with the number of peaks in a multiplet (e.g., singlet, doublet, triplet, quartet) determined by the number of neighboring protons plus one, following the n+1 rule, where 'n' is the number of equivalent neighboring protons.

The coupling constant, denoted as 'J', is the distance between the peaks in a multiplet, measured in Hertz, and provides information about the dihedral angle between the coupled protons, which can be used to determine the three-dimensional conformation of the molecule. The application of computer-assisted structure elucidation software packages can significantly aid in the interpretation of complex NMR spectra, especially when dealing with large and complex organic molecules.[39] Modern NMR spectrometers have become more compact, marking the arrival of desktop and portable instruments capable of addressing intricate tasks such as determining the complex structures of large molecules and conducting industrial, environmental, and food analyses across various solids, solutions, and dispersed systems.[38]

Analyzing the 1H NMR spectrum of efavirenz and its associated impurities involves a systematic approach, starting with the identification of the known signals corresponding to the efavirenz molecule itself, using reference spectra and literature data. Any additional signals observed in the spectrum that do not correspond to efavirenz are indicative of impurities, which then need to be characterized.[43] The chemical shifts of these impurity signals can provide initial clues about the types of functional groups present, such as aromatic rings, alkyl chains, or heteroatoms. For instance, signals in the range of 7-8 ppm typically indicate aromatic protons, while signals in the range of 0-2 ppm are characteristic of aliphatic protons. The

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integration of the impurity signals, relative to the efavirenz signals, allows for the quantitative determination of the amount of each impurity present in the sample. Multiplicity patterns, such as singlets, doublets, triplets, and quartets, provide valuable information about the connectivity of protons within the impurity molecule. Additionally, techniques like COSY and HSQC can be employed to establish connectivity between protons and carbon atoms, respectively, providing further structural information. By systematically analyzing these parameters, it is possible to piece together the structure of the unknown impurities and identify their origin, whether from synthetic byproducts, degradation products, or contaminants. The process becomes challenging and time-consuming because no method reports a complete analysis of total compounds.[78]

NMR spectroscopy can identify the position of functional groups such as methyl groups or halogens on an aromatic ring.[49] Monoterpenes, for example, exhibit diastereotopic hydrogens and methyl groups that are readily identified using 13C-NMR and HETCOR techniques.[46]

The use of 1H NMR spectroscopy in impurity profiling is particularly valuable in the pharmaceutical industry, where stringent regulations require the identification and quantification of all impurities present above a certain threshold. In the case of efavirenz, a non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV/AIDS, the identification and control of impurities are crucial for ensuring the safety and efficacy of the drug product. Understanding the synthetic route and potential degradation pathways of efavirenz is essential for predicting the types of impurities that may be present. Process-related impurities can arise from incomplete reactions, side reactions, or the use of specific reagents or catalysts[68]. Degradation impurities, on the other hand, can form during storage or under stress conditions such as heat, light, or exposure to oxygen or moisture.

By comparing the 1H NMR spectra of different batches of efavirenz, it is possible to identify variations in the impurity profiles and trace them back to specific changes in the manufacturing process or storage conditions. Impurities are unwanted chemicals that persist within a formulation, and identifying, isolating, and characterizing them is an essential aspect of drug development and the pharmaceutical industry. After isolation and identification of impurities, they are characterized using techniques like NMR and LC-MS. The presence of even small amounts of impurities can influence the efficacy and safety of a drug.[1]

Moreover, the application of multivariate data analysis techniques to 1H NMR spectra can enhance the ability to discriminate between different batches of efavirenz and identify subtle differences in their impurity profiles.[79] This approach involves the use of statistical methods to analyze the entire spectrum as a single data point, rather than focusing on individual signals. By combining 1H NMR spectroscopy with other analytical techniques, such as liquid chromatography-mass spectrometry, it is possible to obtain a more comprehensive understanding of the impurity profile of efavirenz and ensure the quality and safety of the drug product.[2] Also, a combination of various spectroscopic techniques like IR, NMR, and mass spectroscopy is used for structural elucidation. For example, IR spectroscopy can reveal the presence of functional groups, while mass spectroscopy can help determine the molecular weight of the impurity. NMR spectra gives the shifts due to N-H, aromatic C-H, aliphatic C-H, and carboxylic O-H. Also, the spectra are recorded at particular frequencies using specific solvents.

HPLC Analysis of Impurities in Efavirenz: High-performance liquid chromatography stands as a pivotal analytical technique for the identification and quantification of impurities within pharmaceutical compounds, ensuring drug safety and efficacy. The presence of impurities in pharmaceutical products can compromise their therapeutic effects and potentially lead to adverse health outcomes, making their detection and control crucial.[6]

HPLC's versatility stems from its ability to separate complex mixtures based on the differential interactions of analytes with a stationary phase and a mobile phase.[32] Efavirenz, a non-nucleoside reverse transcriptase inhibitor widely used in the treatment of human immunodeficiency virus infection, necessitates stringent impurity profiling due to its potential for long-term use and the vulnerability of the patient population it serves. The identification and characterization of these impurities are essential for understanding their formation pathways and implementing effective control strategies during the manufacturing process.[6] Impurity profiling, as emphasized by regulatory bodies, involves identifying and quantifying impurities, some of which are unavoidable even in trace amounts.[1,2] These impurities can arise from various sources, encompassing starting materials, intermediates, by-products of the synthetic route, degradation products formed during storage, and even interactions with the container closure system.[1]

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The development of sensitive and selective HPLC methods is thus paramount for monitoring these impurities and ensuring the quality of efavirenz drug products. The optimization of HPLC methods for efavirenz impurity analysis requires careful consideration of several parameters, including the stationary phase, mobile phase composition, gradient elution program, flow rate, and detection wavelength.[80] Columns with different selectivities, such as C18 or phenyl phases, can be employed to achieve optimal separation of efavirenz and its related impurities. Mobile phases typically consist of mixtures of water, organic modifiers (such as acetonitrile or methanol), and buffering agents to control pH and ionic strength. Gradient elution, where the mobile phase composition is changed over time, is often used to improve the separation of complex mixtures of impurities.

Reversed-phase chromatography, utilizing an RP-C18 column, coupled with an isocratic mobile phase of acetonitrile and acetic acid aqueous solution, demonstrates efficacy in separating and detecting impurities, with UV detection at 280 nm.[81] The flow rate also influences separation efficiency and analysis time. Furthermore, the detection wavelength should be carefully selected to maximize the sensitivity for both efavirenz and its impurities, often involving scanning the UV spectrum to identify the wavelength of maximum absorbance. It is worth noting that during the synthesis of other pharmaceutical compounds like ezetimibe, process-related impurities have been detected and characterized using HPLC, highlighting the importance of this technique in monitoring the quality of drug substances.[67,68]

The emergence of ultra-performance liquid chromatography offers significant advantages over traditional HPLC, including reduced run times, lower solvent consumption, and enhanced peak resolution.[82] This is particularly relevant in the pharmaceutical industry, where there is a growing demand for faster and more efficient analytical methods.[10] The use of sub-2 μm particle size packed columns in UHPLC allows for separations at ultra-high pressures, leading to improved resolution and sensitivity. Moreover, nano-liquid chromatography and capillary electrochromatography offer high selectivity, separation efficiency, and resolution, with short analysis times and low mobile phase consumption.[33]

Hyphenated techniques, such as LC-MS and LC-MS/MS, provide enhanced selectivity and sensitivity for impurity detection and identification. LC-MS/MS has become the method of choice in recent years. Mass spectrometry detection allows for the determination of the molecular weight of separated compounds, aiding in the identification of unknown impurities.

The development of automated sample extraction and clean-up methodologies further reduces sample manipulation, variability, and total analysis time. On-line solid-phase extraction coupled to HPLC or UHPLC methods is beneficial.[29] These advancements contribute to more robust and reliable impurity profiling of efavirenz, ultimately ensuring drug product quality and patient safety.

CHALLENGES AND FUTURE DIRECTIONS:
Navigating the Complexities of Impurity Profiling-Challenges and Considerations: Impurity profiling, the comprehensive characterization of identified and unidentified impurities within drug products, stands as a critical pillar in ensuring pharmaceutical safety and efficacy.[2] This intricate process involves a multifaceted approach, encompassing method development, validation, and adherence to stringent regulatory requirements. The presence of impurities, even in trace amounts, can potentially compromise the therapeutic effect of a drug and pose significant health risks to patients, necessitating their careful monitoring and control. Impurities are unwanted chemicals that can arise from various sources, including the raw materials used in synthesis, the manufacturing process itself, degradation during storage, or interactions with excipients in the formulation.[1] Pharmaceutical products are often produced through total synthesis or modification of natural products, leading to a range of organic substances that can persist as impurities in the final product.[3]

The initial hurdle in impurity profiling lies in the development of robust and sensitive analytical methods capable of detecting, quantifying, and identifying these unwanted components.[2] Liquid chromatography coupled with mass spectrometry and gas chromatography coupled with mass spectrometry are indispensable tools for impurity profiling, offering high sensitivity and selectivity for a wide range of chemical compounds.[6] These techniques enable the separation and identification of impurities based on their physicochemical properties and mass-to-charge ratios. Selecting the appropriate chromatographic conditions, such as the stationary phase, mobile phase, and gradient program, is crucial for achieving adequate separation of impurities from the active pharmaceutical ingredient and other formulation components. Sample preparation techniques, such as extraction, filtration, and concentration, play a critical role in isolating and enriching impurities from the drug product matrix, thereby enhancing their detectability. The development of effective

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methods for impurity profiling is vital because any pharmaceutical product should be free from impurities in order to serve its intended therapeutic activity.[5] At the early stages of drug development, it is essential to separate a relatively large number of process-related impurities, synthetic intermediates, and degradation products to characterize starting materials and products of chemical synthesis.[10]

Method validation is an indispensable step in ensuring the reliability and accuracy of impurity profiling data. Validation involves systematically assessing the performance characteristics of the analytical method, including its selectivity, sensitivity, linearity, accuracy, precision, and robustness. Selectivity refers to the ability of the method to distinguish and quantify the target impurities in the presence of other components in the sample matrix. Sensitivity reflects the lowest concentration of an impurity that can be reliably detected and quantified. Linearity establishes the concentration range over which the method provides a proportional response. Accuracy assesses the closeness of the measured values to the true values, while precision evaluates the reproducibility of the measurements. Robustness evaluates the method's capacity to remain unaffected by small, but deliberate variations in method parameters.[7] Meeting regulatory requirements for method validation is imperative for ensuring the acceptability of impurity profiling data for regulatory submissions.

Stringent regulatory guidelines, such as those established by the International Council for Harmonisation, govern the acceptable levels of impurities in pharmaceutical products.[2] These guidelines provide a framework for identifying, characterizing, and controlling impurities to ensure patient safety. The guidelines outline specific analytical procedures that must be validated, including identification tests, quantitative tests for impurities content, and limit tests for the control of impurities.[83] These guidelines also define thresholds for impurities based on their potential toxicity, requiring that impurities exceeding these thresholds be identified and characterized. The ICH guidelines provide a harmonized approach to impurity control, facilitating the global development and registration of pharmaceutical products. Analytical method validation gives information about various parameters like accuracy, precision, linearity, Limit of Detection, Limit of Quantification, specificity, range and robustness, and validation should be done as per regulatory guidelines such as ICH guidelines ("Systematic Reviews in Pharmacy," 2020). Agencies scrutinize impurity guidelines for the safety assessment and control of small-molecule drugs.[4]

A significant challenge in impurity profiling lies in obtaining or synthesizing reference standards for all identified impurities. Reference standards are highly purified materials used to calibrate analytical methods and quantify the levels of impurities in drug products.[84] The synthesis of impurities can be particularly challenging for complex molecules or unstable compounds, requiring expertise in synthetic chemistry and purification techniques. In certain instances, obtaining reference standards from commercial sources may be difficult or impossible, necessitating in-house synthesis or isolation from the drug product. Without authentic standards, accurate quantification becomes problematic, potentially leading to inaccurate risk assessments. The isolation and characterization of new degradation products can be particularly challenging, requiring advanced analytical techniques and expertise in structural elucidation.

The absence of a structure elucidation of an impurity in drug products is explicitly accommodated in regulatory guidance.[9] Mutagenic impurities represent a distinct category of impurities that require special attention due to their potential to cause DNA damage and increase the risk of cancer.[8] These impurities, even at very low concentrations (ppm levels), must be carefully controlled to minimize the potential for adverse health effects.[11] Highly sensitive analytical methods are required to detect and quantify these impurities, often involving techniques such as liquid chromatography coupled with tandem mass spectrometry. Identifying and controlling mutagenic impurities often requires collaboration between analytical chemists, toxicologists, and regulatory experts. Impurity profiling is a critical aspect of pharmaceutical development and quality control, ensuring the safety and efficacy of drug products.[1] Thorough toxicological evaluation is essential to determine the permissible levels of degradation products, especially when genotoxic impurities are detected.[69] It is important to differentiate between "establishment of purity" and "searching for impurities" to define purity as a determinant of drug substance and product quality, efficacy and safety.[85] Impurities are unwanted chemicals that remain with the active pharmaceutical ingredient or develop during formulation; they may be identified or unidentified, and intrinsic or extrinsic.[69,70] In the early phases of drug development, the cost of labor is much greater than the cost of raw materials.[12,86]

Emerging Techniques and Technologies for Impurity Profiling: The landscape of pharmaceutical analysis is undergoing a significant transformation with the advent of innovative techniques and technologies that are revolutionizing impurity

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profiling.[7] Impurity profiling, the process of identifying and quantifying impurities in drug substances and products, is a critical aspect of pharmaceutical development and quality control, ensuring drug safety and efficacy.[1,2] As pharmaceutical products age during their shelf life, their purity changes, making purity a key determinant of drug substance and product quality.[85] Traditional methods, while reliable, often face limitations in terms of sensitivity, resolution, and efficiency, spurring the development of more advanced approaches. Capillary electrophoresis has emerged as a powerful separation technique that offers high resolution and sensitivity for the analysis of charged impurities. Mass spectrometry, particularly when coupled with liquid chromatography, has become indispensable for the identification and characterization of impurities, providing structural information and enabling the detection of trace levels of contaminants.[7] Raman spectroscopy is a vibrational spectroscopy technique, which provides structural information and is used for identifying polymorphs, hydrates, and salts.[22] Chromatography is used to separate these complex mixtures.[10]

Developments in chromatographic techniques have significantly impacted the field of impurity profiling.[26] Ultra-high-performance liquid chromatography, with its sub-2 μm particle size columns, enables high-resolution separations with reduced analysis times, facilitating the rapid identification and quantification of impurities.[29] Advanced mass spectrometry techniques, such as high-resolution mass spectrometry, offer enhanced accuracy and sensitivity for impurity detection and structural elucidation.[76] Furthermore, the integration of chemometrics and data analytics tools has streamlined the analysis of complex chromatographic and spectroscopic data, enabling more efficient impurity identification and quantification. These advancements collectively contribute to a more comprehensive and efficient approach to impurity profiling, ensuring the safety and efficacy of pharmaceutical products.[30] Liquid chromatography is a critical technique used for purifying therapeutic drugs on a large scale. The scientific fundamentals of the underlying phenomena require a better understanding for enhanced quality of life.[87] Laser-induced fluorescence is a very sensitive technique for qualitative and quantitative analysis.[32]

The integration of mass spectrometry with separation techniques like liquid chromatography has become a cornerstone of modern impurity profiling, particularly with the introduction of high-resolution mass analyzers in hybrid configurations.[28,88] High-resolution mass spectrometry excels in the evaluation of complex mixtures, accurately determining the mass and chemical structure of compounds within a sample.[89] Non-chromatographic techniques, such as immunoassay using monoclonal antibodies, phytochemical screening assays, and Fourier-transform infrared spectroscopy, can also be employed to obtain and facilitate the identification of bioactive compounds.[90] The fusion of advanced separation methods with mass spectrometry provides unparalleled capabilities for comprehensive impurity profiling, meeting the stringent requirements of modern pharmaceutical analysis. These approaches facilitate the detection and identification of impurities, even when present in complex matrices or at low concentrations.[25] In addition to chromatography-based approaches, direct MS techniques are available which offer direct sample examination and high sample throughput.[31]

Affinity chromatography is a purification technique.[52] This technique is also called bio-specific adsorption chromatography. This process separates proteins through non-covalent interactions with a specific ligand. The ligand is immobilized to a solid support such as a resin.[91] Other chromatography techniques are based on the stationary bed, including column, thin layer, and paper chromatography. Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.[63] The target protein is captured by the affinity ligand while other molecules are washed away. The interaction between the target protein and the ligand can be disrupted by changing the buffer conditions such as pH, ionic strength, or adding a competitive molecule.

CONCLUSION:

Impurity Profiling - A Critical Step in Ensuring Pharmaceutical Quality and Safety: The presence of impurities in pharmaceutical products is an unavoidable reality, stemming from the inherent complexities of chemical synthesis, manufacturing processes, and degradation pathways.[1] These impurities, even in trace amounts, can significantly impact the efficacy and safety of drug products, potentially leading to adverse effects and compromising patient well-being.[1,3] Impurity profiling, a comprehensive analytical process, plays a crucial role in identifying, characterizing, and quantifying these unwanted substances in both drug substances and drug products.[1,2] This process is essential for ensuring the quality, safety, and efficacy of pharmaceuticals throughout their lifecycle.[85] Structural identification of an impurity is paramount to understanding its formation and devising effective

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control strategies.[6] The raw materials used in drug synthesis often have lower quality standards compared to the final drug substance, making them potential sources of impurities.[2] Impurity profiling helps in understanding the origin and fate of these impurities during the manufacturing process[6]. Ultimately, the goal is to minimize their presence in the final product.[1]

The International Council for Harmonisation guidelines provide a harmonized framework for the safety assessment and control of impurities in pharmaceuticals.[2] These guidelines offer a structured approach to identify and manage impurities, ensuring patient safety.[4] These guidelines acknowledge that impurities with unidentified structures can also pose risks and should be carefully evaluated.[9] The identification of impurities is critical, but their quantification is equally important.[5] The levels of impurities must be carefully controlled within acceptable limits to ensure that the drug product meets predefined quality standards. Impurity profiling is not just a regulatory requirement but a fundamental scientific endeavor. It drives innovation in analytical techniques, separation science, and detection methods. The information generated during impurity profiling helps optimize manufacturing processes, reducing the formation of unwanted byproducts.

Pharmaceutical development relies heavily on impurity profiling to guarantee the excellence and safety of medications.[7] It is essential to identify, measure, and control impurities in both drug substances and drug products.[70] Early in the development process, a wide array of process-related impurities, synthetic intermediates, and degradation products must be separated to characterize starting materials and the products of chemical synthesis.[10] Comprehensive impurity profiling helps determine the fate and carry-over of impurities from raw materials to the final drug product.[12] It provides a basis for setting appropriate specifications and acceptance criteria, ensuring that the drug product consistently meets quality standards throughout its shelf life. Analytical methods used in impurity profiling, such as high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance, are constantly evolving to provide more sensitive and selective detection of impurities.[92]

The pharmaceutical industry has adopted quality by design and process analytical technology to improve drug product quality.[93] These strategies facilitate the optimization of analytical methods, enabling better impurity detection and quantification.[94] Risk assessments, based on data gathered during impurity profiling, help in identifying critical process parameters and control points that can influence impurity levels.[95] Continuous monitoring and control strategies can be implemented to ensure consistent product quality. Impurity profiling plays a crucial role in ensuring the safety of drug products by identifying and quantifying potentially toxic impurities. Genotoxic impurities, for example, are of particular concern because they can cause DNA damage and potentially lead to cancer.[8] Therefore, impurity profiling helps in setting safe limits for these impurities, protecting patients from potential harm.[11] By understanding the degradation pathways of drug substances and drug products, impurity profiling can help in developing more stable formulations and packaging. Impurities can arise from various sources, including raw materials, manufacturing processes, degradation of the active pharmaceutical ingredient, or interactions with excipients or packaging materials.[69,96]

Effective cleaning procedures are essential in pharmaceutical manufacturing to prevent product contamination. Cleaning validation ensures the removal of contaminants from previous products, cleaning agent residues, and microbial contaminants. Impurity profiling also helps in assessing the effectiveness of cleaning procedures by monitoring for residual impurities.[73]

In summary, impurity profiling is an indispensable tool in the pharmaceutical industry. It contributes significantly to the development of safe and effective drug products, safeguarding public health. By providing a comprehensive understanding of the impurity landscape, impurity profiling enables pharmaceutical manufacturers to make informed decisions, optimize manufacturing processes, and ensure the consistent quality of their products.[7,97] Ultimately, it is a critical component of pharmaceutical quality assurance.[69,72,98]

The Role of LCMS, 1H NMR, and HPLC in Impurity Profiling: A Case Study of Efavirenz: Impurity profiling stands as a critical facet of pharmaceutical analysis, ensuring drug products' safety and efficacy by identifying, quantifying, and characterizing extraneous substances that may arise during synthesis, formulation, or storage.[6] These impurities, even in trace amounts, can significantly impact a drug's therapeutic effect and safety profile, necessitating rigorous analytical techniques for their detection and control.[1,2,6] Regulatory bodies like the International Council for Harmonisation mandate the identification and control of impurities to ensure that drug products meet stringent quality standards.[1] Among the array of analytical methodologies employed, liquid chromatography-mass spectrometry, proton nuclear magnetic resonance (^{1}H NMR), and high-performance liquid

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chromatography have emerged as indispensable tools for impurity profiling, offering complementary capabilities in terms of sensitivity, selectivity, and structural elucidation.[7,22,25]

Liquid chromatography-mass spectrometry has revolutionized impurity profiling due to its ability to separate, detect, and identify compounds with high sensitivity and specificity.[23] This technique combines the separation power of liquid chromatography with the detection capabilities of mass spectrometry, enabling the analysis of complex mixtures and the identification of unknown impurities based on their mass-to-charge ratio.[26] In LC-MS, the liquid chromatography component separates the components of a mixture based on their physical and chemical properties, while the mass spectrometry component measures the mass-to-charge ratio of the separated components. The combination of these two techniques provides a powerful tool for identifying and quantifying impurities in pharmaceutical products.[28] LC-MS/MS provides a reliable alternative because it is sensitive enough to quantify toxicologically relevant levels.[35] The fragmentation patterns obtained through tandem mass spectrometry provide valuable structural information, aiding in the identification of unknown impurities by comparing them with reference standards or through *de novo* structural elucidation.[24] High-resolution mass spectrometry, such as quadrupole-time-of-flight and linear ion trap-orbitrap technology, further enhances the reliability of impurity identification, enabling accurate mass measurements and molecular formula assignment, thereby increasing selectivity against the matrix background.[76] Nonetheless, advanced automated software solutions, enhanced prediction systems for theoretical fragmentation patterns, and retention times are needed for unknown compounds' structure elucidation within a reasonable time frame and with reasonable soundness.

Proton nuclear magnetic resonance spectroscopy serves as a complementary technique to LC-MS in impurity profiling, providing detailed structural information about impurities based on the magnetic properties of atomic nuclei. 1H NMR spectroscopy is a powerful analytical technique that provides detailed information about the structure and environment of hydrogen atoms in a molecule. By analyzing the chemical shifts, splitting patterns, and integration values of the signals in the 1H NMR spectrum, it is possible to elucidate the structure of the molecule, including the identification of functional groups and the connectivity of atoms. This non-destructive technique allows for the identification and quantification of impurities without the need for extensive sample preparation, providing valuable insights into their chemical structures and stereochemistry. The combination of 1H NMR with other spectroscopic techniques, such as 13C NMR, COSY, HSQC, and HMBC, can provide even more detailed structural information, enabling the complete characterization of impurities. Moreover, NMR spectroscopy can be used to determine the purity of a compound by comparing the integral of the signals of the compound of interest to the integral of the signals of the impurities.

High-performance liquid chromatography remains a cornerstone technique in impurity profiling, offering high resolution and quantitative capabilities for separating and quantifying impurities in pharmaceutical samples. HPLC is a separation technique that separates the components of a mixture based on their interactions with a stationary phase and a mobile phase. HPLC is extensively employed for separating, identifying, and quantifying each of the components in a mixture. Various detectors can be coupled with HPLC, including UV-Vis, fluorescence, refractive index, and mass spectrometry, each offering different sensitivity and selectivity depending on the properties of the impurities being analyzed.[32] Gradient elution techniques in HPLC optimize the separation of impurities with varying polarities, while techniques like chiral HPLC enable the separation of enantiomeric impurities.[52] The choice of stationary phase, mobile phase, and detection method can be optimized to achieve the desired resolution and sensitivity for the separation and quantification of impurities. HPLC is applied to several categories of substances such as carbohydrates, lipids, vitamins, additives, synthetic colorings, natural pigments, contaminants, and amino acids.[34]

These techniques are used to control the purity of proteins, amino acids, nucleic acids, hydrocarbons, carbohydrates, drugs, antibiotics, and steroids.[63]

In the specific context of Efavirenz, these analytical techniques play a crucial role in ensuring the quality and safety of the drug product by detecting and characterizing potential impurities that may arise during synthesis, formulation, or storage. Impurity profiling of Efavirenz involves the identification, quantification, and structural elucidation of impurities present in the drug substance or product. LC-MS is used to identify and quantify the impurities present in Efavirenz samples, while 1H NMR spectroscopy provides detailed structural information about the identified impurities. HPLC is employed for the separation and quantification of impurities in Efavirenz samples, ensuring the drug product meets stringent quality standards and regulatory requirements.

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Different analytical techniques, most of which depend on separation techniques such as high-performance liquid chromatography, gas-liquid chromatography, supercritical fluid chromatography, and capillary electrophoresis, can be used to identify impurities in pharmaceuticals.[7] Impurities profiling is a critical aspect of pharmaceutical development and quality control, ensuring drug products' safety and efficacy.[90] LC-MS, 1H NMR, and HPLC each offer unique advantages and play complementary roles in impurity profiling, providing comprehensive information on the identity, structure, and quantity of impurities, which is crucial for ensuring the quality and safety of pharmaceutical products like Efavirenz.

REFERENCE:

1. Thakur, P. (2020) "isolation of pharmaceutical impurities from bulk drug preparations," International Journal of Advanced Research, 8(6), p. 1436. doi:10.21474/ijar01/11232.
2. Dhangar, K. et al. (2017) "impurity profiling of drugs towards safety and efficacy: theory and practice," Journal of the Chilean Chemical Society, 62(2), p. 3543. doi:10.4067/s0717-97072017000200024.
3. Mammone, F.R. et al. (2023) "A Novel Validated UHPLC Method for the Estimation of Rosuvastatin and Its Complete Impurity Profile in Tablet Formulations," Molecules, 28(1), p. 431. doi:10.3390/molecules28010431.
4. Snodin, D.J. and McCrossen, S. (2012) "Guidelines and pharmacopoeial standards for pharmaceutical impurities: Overview and critical assessment," Regulatory Toxicology and Pharmacology. Elsevier BV, p. 298. doi:10.1016/j.yrtph.2012.03.016.
5. Parmar, I., Rathod, H.K.K. and Shaik, S. (2021) "A Review: Recent Trends in Analytical Techniques for Characterization and Structure Elucidation of Impurities in the Drug Substances," Indian Journal of Pharmaceutical Sciences, 83(3). doi:10.36468/pharmaceutical-sciences.789.
6. Qiu, F. et al. (2009) "Identification of a process impurity formed during synthesis of a nevirapine analogue HIV NNRT inhibitor using LC/MS and forced degradation studies," Journal of Pharmaceutical and Biomedical Analysis, 49(3), p. 733. doi:10.1016/j.jpba.2009.01.010.
7. Victoria, F. (2014) "Various Aspects in the Impurity Profiling of Pharmaceutical Formulations," Pharmaceutica Analytica Acta, 5(8). doi:10.4172/2153-2435.1000e163.
8. Charoo, N.A. et al. (2019) "Lesson Learnt from Recall of Valsartan and Other Angiotensin II Receptor Blocker Drugs Containing NDMA and NDEA Impurities," AAPS PharmSciTech, 20(5). doi:10.1208/s12249-019-1376-1.
9. Abdin, A.Y., Yeboah, P. and Jacob, C. (2020) "Chemical Impurities: An Epistemological Riddle with Serious Side Effects," International Journal of Environmental Research and Public Health. Multidisciplinary Digital Publishing Institute, p. 1030. doi:10.3390/ijerph17031030.
10. Szücs, R. et al. (2017) "Liquid chromatography in the pharmaceutical industry," in Elsevier eBooks. Elsevier BV, p. 515. doi:10.1016/b978-0-12-805392-8.00017-7.
11. Nordström, F.L. et al. (2023) "Prevalence of Impurity Retention Mechanisms in Pharmaceutical Crystallizations," Organic Process Research & Development, 27(4), p. 723. doi:10.1021/acs.oprd.3c00009.
12. Pathy, K. (2018) "Considerations for Successful Scale-Up to Tox Batches and Phase-Api (Bulk Drug substance)," Biomedical Journal of Scientific & Technical Research, 8(3). doi:10.26717/bjstr.2018.08.001662.
13. Castelnuovo, B. et al. (2016) "First-line antiretroviral therapy durability in a 10-year cohort of naïve adults started on treatment in Uganda," Journal of the International AIDS Society, 19(1). doi:10.7448/ias.19.1.20773.
14. Staszewski, S. et al. (1999) "Efavirenz plus Zidovudine and Lamivudine, Efavirenz plus Indinavir, and Indinavir plus Zidovudine and Lamivudine in the Treatment of HIV-1 Infection in Adults," New England Journal of Medicine, 341(25), p. 1865. doi:10.1056/nejm199912163412501.
15. Weber, I.T., Wang, Y. and Harrison, R.W. (2021) "HIV Protease: Historical Perspective and Current Research," Viruses. Multidisciplinary Digital Publishing Institute, p. 839. doi:10.3390/v13050839.
16. Doxey, C.H. (2021) "Receiving." doi:10.1002/9781119700548.ch10.
17. Gregson, J. et al. (2016) "Global epidemiology of drug resistance after failure of WHO recommended first-line regimens for adult HIV-1 infection: a multicentre retrospective cohort study," The Lancet Infectious Diseases, 16(5), p. 565. doi:10.1016/s1473-3099(15)00536-8.
18. Atta, M.G., Seigneux, S. de and Lucas, G.M. (2018) "Clinical Pharmacology in HIV Therapy," Clinical Journal of the American Society of Nephrology. Lippincott Williams & Wilkins, p. 435. doi:10.2215/cjn.02240218.
19. Kalapila, A.G. and Marrazzo, J. (2016) "Antiretroviral Therapy for Prevention of Human Immunodeficiency Virus Infection," Medical Clinics of North America. Elsevier BV, p. 927. doi:10.1016/j.mcna.2016.03.013.
20. López-Cortés, L.F. et al. (2002) "Pharmacokinetic Interactions Between Efavirenz and Rifampicin in HIV-Infected Patients with Tuberculosis," Clinical Pharmacokinetics, 41(9), p. 681. doi:10.2165/00003088-200241090-00004.
21. Kuwahara, T., Yoshino, M. and Shirasaka, T. (2007) "Adverse Effects of Antiretroviral Agents," The Journal of AIDS Research, 9(3), p. 191. doi:10.11391/aidsr1999.9.191.
22. Narayanam, M. et al. (2013) "Critical practical aspects in the application of liquid chromatography–mass spectrometric studies for the characterization of impurities and degradation products," Journal of Pharmaceutical and Biomedical Analysis. Elsevier BV, p. 191. doi:10.1016/j.jpba.2013.04.027.
23. Bulska, E. et al. (2020) "Comprehensive Protocol for the Identification and Characterization of New Psychoactive Substances in the Service of Law Enforcement Agencies," Frontiers in Chemistry, 8. doi:10.3389/fchem.2020.00693.
24. Li, B. et al. (2009) "Characterization of impurities in tobramycin by liquid chromatography–mass spectrometry," Journal of Chromatography A, 1216(18), p. 3941. doi:10.1016/j.chroma.2009.03.023.
25. Steinmann, D. and Ganzera, M. (2010) "Recent advances on HPLC/MS in medicinal plant analysis," Journal of Pharmaceutical and Biomedical Analysis, 55(4), p. 744. doi:10.1016/j.jpba.2010.11.015.
26. Yao, S. et al. (2010) "Ten Years of Research into Phytomedicines Analysis - An Era in New Technologies and Methods," Current Pharmaceutical Analysis, 6(4), p. 269.

doi:10.2174/157341210793292383
27. Ashraf, S.A. et al. (2020) "UPLC-MS: An Emerging Novel Technology and Its Application in Food Safety," in IntechOpen eBooks. IntechOpen. doi:10.5772/intechopen.92455
28. Mattarozzi, M. and Careri, M. (2015) "Liquid Chromatography/Mass Spectrometry in Environmental Analysis," Encyclopedia of Analytical Chemistry. doi:10.1002/9780470027318.a0840.pub2.
29. Núñez, Ó. et al. (2013) "State-of-the-art in fast liquid chromatography–mass spectrometry for bio-analytical applications," Journal of Chromatography B. Elsevier BV, p. 3. doi:10.1016/j.jchromb.2012.12.031.
30. Vázquez-Martínez, J. and López, M.G. (2019) "Chemometrics-Based TLC and GC-MS for Small Molecule Analysis: A Practical Guide," in IntechOpen eBooks. IntechOpen. doi:10.5772/intechopen.77160.
31. Václavík, L. et al. (2014) "Survey of Mass Spectrometry-Based High-Throughput Methods in Food Analysis." doi:10.1002/9781118907924.ch02.
32. Patil, A. et al. (2012) "Highly Sensitive High Performance Liquid Chromatography-Laser Induced Fluorescence for Proteomics Applications," ISRN Spectroscopy, 2012, p. 1. doi:10.5402/2012/643979.
33. Fanali, S. (2017) "An overview to nano-scale analytical techniques: Nano-liquid chromatography and capillary electrochromatography," Electrophoresis. Wiley, p. 1822. doi:10.1002/elps.201600573.
34. Bélanger, J.M.R., Paré, J.R.J. and Sigouin, M. (1997) "Chapter 2 High performance liquid chromatography (HPLC): Principles and applications," in Techniques and instrumentation in analytical chemistry. CRC Press, p. 37. doi:10.1016/s0167-9244(97)80011-x.
35. Holderied, I., Rychlik, M. and Elsinghorst, P.W. (2019) "Optimized Analysis of Ergot Alkaloids in Rye Products by Liquid Chromatography-Fluorescence Detection Applying Lysergic Acid Diethylamide as an Internal Standard," Toxins, 11(4), p. 184. doi:10.3390/toxins11040184.
36. Ridgway, K., Lalljie, S.P.D. and Smith, R.M. (2007) "Sample preparation techniques for the determination of trace residues and contaminants in foods," Journal of Chromatography A. Elsevier BV, p. 36. doi:10.1016/j.chroma.2007.01.134.
37. Cappiello, A. et al. (2008) "Overcoming Matrix Effects in Liquid Chromatography−Mass Spectrometry," Analytical Chemistry, 80(23), p. 9343. doi:10.1021/ac8018312.
38. Кужаева, А.А., Zgonnik, P.V. and Анисимова, Н.А. (2019) "Nuclear magnetic resonance methods – the key to determining the structure of organic substances," Journal of Physics Conference Series, 1384(1), p. 12071. doi:10.1088/1742-6596/1384/1/012071.
39. Soong, R. et al. (2020) "CASE (Computer-Assisted Structure Elucidation) Study for an Undergraduate Organic Chemistry Class," Journal of Chemical Education, 97(3), p. 855. doi:10.1021/acs.jchemed.9b00498.
40. Tampieri, A. et al. (2020) "A brief introduction to the basics of NMR spectroscopy and selected examples of its applications to materials characterization," Physical Sciences Reviews, 6(1). doi:10.1515/psr-2019-0086.
41. Grover, V.P.B. et al. (2015) "Magnetic Resonance Imaging: Principles and Techniques: Lessons for Clinicians," Journal of Clinical and Experimental Hepatology. Elsevier BV, p. 246. doi:10.1016/j.jceh.2015.08.001.
42. Arora, H. (2018) "Review of basic Concepts involved in Magnetic Resonance Imaging," Biomedical Journal of Scientific & Technical Research, 8(5). doi:10.26717/bjstr.2018.08.001726.
43. Emwas, A. et al. (2014) "Standardizing the experimental conditions for using urine in NMR-based metabolomic studies with a particular focus on diagnostic studies: a review," Metabolomics. Springer Science+Business Media, p. 872. doi:10.1007/s11306-014-0746-7.
44. Fulmer, G.R. et al. (2010) "NMR Chemical Shifts of Trace Impurities: Common Laboratory Solvents, Organics, and Gases in Deuterated Solvents Relevant to the Organometallic Chemist," Organometallics, 29(9), p. 2176. doi:10.1021/om100106e.
45. Renner, G., Schmidt, T. and Schram, J. (2016) "Characterization and Quantification of Microplastics by Infrared Spectroscopy," in Comprehensive analytical chemistry. Elsevier BV, p. 67. doi:10.1016/bs.coac.2016.10.006.
46. Alty, L.T. (2005) "Monoterpene Unknowns Identified Using IR, 1H-NMR, 13C-NMR, DEPT, COSY, and HETCOR," Journal of Chemical Education, 82(9), p. 1387. doi:10.1021/ed082p1387.
47. Booker, A. et al. (2016) "From Traditional Resource to Global Commodities:—A Comparison of Rhodiola Species Using NMR Spectroscopy—Metabolomics and HPTLC," Frontiers in Pharmacology, 7. doi:10.3389/fphar.2016.00254.
48. Peterson, D.J. and Loening, N.M. (2007) "QQ-HSQC: a quick, quantitative heteronuclear correlation experiment for NMR spectroscopy," Magnetic Resonance in Chemistry, 45(11), p. 937. doi:10.1002/mrc.2073.
49. Duffy, J. et al. (2018) "Differentiation of fentanyl analogues by low-field NMR spectroscopy," Analytica Chimica Acta, 1049, p. 161. doi:10.1016/j.aca.2018.12.014.
50. Hu, J.Z., Xiao, X. and Hu, M.Y. (2016) "NMR Metabolomics in Ionizing Radiation.," PubMed, 1. Available at: https://pubmed.ncbi.nlm.nih.gov/28707005 (Accessed: February 2025).
51. Huck, C.W. (2015) "Infrared Spectroscopic Technologies for the Quality Control of Herbal Medicines," in Elsevier eBooks. Elsevier BV, p. 477. doi:10.1016/b978-0-12-800874-4.00022-2.
52. Wen, Q. et al. (2023) "Production Technology and Functionality of Bioactive Peptides," Current Pharmaceutical Design. Bentham Science Publishers, p. 652. doi:10.2174/1381612829666230201121353.
53. Boiteau, R. et al. (2018) "Structure Elucidation of Unknown Metabolites in Metabolomics by Combined NMR and MS/MS Prediction," Metabolites, 8(1), p. 8. doi:10.3390/metabo8010008.
54. McLafferty, F.W. (2008) "Mass spectrometry across the sciences," Proceedings of the National Academy of Sciences, 105(47), p. 18088. doi:10.1073/pnas.0800784105.
55. Reuhs, B.L. and Rounds, M.A. (2010) "High-Performance Liquid Chromatography," in Food science text series. Springer Nature, p. 499. doi:10.1007/978-1-4419-1478-1_28.
56. Galant, A., Kaufman, R.C. and Wilson, J.D. (2015) "Glucose: Detection and analysis," Food Chemistry. Elsevier BV, p. 149. doi:10.1016/j.foodchem.2015.04.071.
57. Shi, Y. et al. (2022) "Recent Progress in the Study of Taste Characteristics and the Nutrition and Health Properties of Organic Acids in Foods," Foods. Multidisciplinary Digital Publishing Institute, p. 3408. doi:10.3390/foods11213408.
58. Wu, N. and Thompson, R.A. (2006) "Fast and Efficient Separations Using Reversed Phase Liquid Chromatography," Journal of Liquid Chromatography &amp Related Technologies, 29, p. 949. doi:10.1080/10826070600574903.

59. Croley, T.R. (2010) "Ultra-Performance Liquid Chromatography–Mass Spectrometry of Proteins," Methods in molecular biology, p. 419. doi:10.1007/978-1-60761-913-0_23.
60. Caleb, J. et al. (2020) "Dispersive liquid-liquid microextraction for the isolation and HPLC-DAD determination of three major capsaicinoids in Capsicum annuum L.," TURKISH JOURNAL OF CHEMISTRY [Preprint]. doi:10.3906/kim-2009-55
61. Snyder, L.R., Kirkland, J.J. and Glajch, J.L. (1997) Practical HPLC Method Development. doi:10.1002/9781118592014.
62. Boysen, R.I. and Hearn, M.T.W. (2010) "High Performance Liquid Chromatographic Separation Methods," in Elsevier eBooks. Elsevier BV, p. 5. doi:10.1016/b978-008045382-8.00183-0.
63. Coşkun, Ö. (2016) "Separation Tecniques: CHROMATOGRAPHY," Northern Clinics of Istanbul. KARE Publishing. doi:10.14744/nci.2016.32757.
64. Goswami, J. (2015) "Different Separation or Experimental Techniques for Clinical Chromatography: Small Review," Journal of Chromatography & Separation Techniques, 6(7). doi:10.4172/2157-7064.1000297.
65. Poole, C.F. (2003) "Laboratory-Scale Preparative Chromatography," in Elsevier eBooks. Elsevier BV, p. 847. doi:10.1016/b978-044450198-1/50024-0.
66. Graumann, K. and Ebenbichler, A.A. (2005) "Development and Scale up of Preparative HIC for the Purification of a Recombinant Therapeutic Protein," Chemical Engineering & Technology, 28(11), p. 1398. doi:10.1002/ceat.200500165.
67. Raman, B. et al. (2009) "Structural elucidation of a process-related impurity in ezetimibe by LC/MS/MS and NMR," Journal of Pharmaceutical and Biomedical Analysis, 52(1), p. 73. doi:10.1016/j.jpba.2009.12.021.
68. Guntupalli, S. et al. (2013) "Identification, isolation and characterization of process related impurities in ezetimibe," Journal of Pharmaceutical and Biomedical Analysis, 88, p. 385. doi:10.1016/j.jpba.2013.09.020.
69. Melo, S.R. de et al. (2014) "Advice on Degradation Products in Pharmaceuticals: A Toxicological Evaluation," PDA Journal of Pharmaceutical Science and Technology. Parenteral Drug Association, p. 221. doi:10.5731/pdajpst.2014.00974.
70. Alvarenga, B.R. de and Carneiro, R.L. (2019) "Chemometrics Approaches in Forced Degradation Studies of Pharmaceutical Drugs," Molecules. Multidisciplinary Digital Publishing Institute, p. 3804. doi:10.3390/molecules24203804.
71. Singh, S. et al. (2013) "Forced degradation studies to assess the stability of drugs and products," TrAC Trends in Analytical Chemistry, 49, p. 71. doi:10.1016/j.trac.2013.05.006.
72. Murtaza, G. et al. (2021) "A Review on the Microbial Contamination in the Non-sterile Pharmaceutical Products," Pharmaceutical Science and Technology. Science Publishing Group, p. 68. doi:10.11648/j.pst.20210502.17.
73. Prabu, S.L., Prakash, T.N.K.S. and Thirumurugan, R. (2014) "Cleaning Validation and Its Regulatory Aspects in the Pharmaceutical Industry," in Elsevier eBooks. Elsevier BV, p. 129. doi:10.1016/b978-0-323-31303-2.00005-4.
74. Rathore, N. and Rajan, R.S. (2008) "Current Perspectives on Stability of Protein Drug Products during Formulation, Fill and Finish Operations," Biotechnology Progress. American Chemical Society, p. 504. doi:10.1021/bp070462h.
75. Ladwig, P.M., Barnidge, D.R. and Willrich, M.A.V. (2017) "Mass Spectrometry Approaches for Identification and Quantitation of Therapeutic Monoclonal Antibodies in the Clinical Laboratory," Clinical and Vaccine Immunology. American Society for Microbiology. doi:10.1128/cvi.00545-16.
76. Krauß, M., Singer, H. and Hollender, J. (2010) "LC–high resolution MS in environmental analysis: from target screening to the identification of unknowns," Analytical and Bioanalytical Chemistry, 397(3), p. 943. doi:10.1007/s00216-010-3608-9.
77. Valenzuela-Gloria, M.S. et al. (2021) "Molecular Characterization of Fungal Pigments," Journal of Fungi. Multidisciplinary Digital Publishing Institute, p. 326. doi:10.3390/jof7050326.
78. Ouyang, H. et al. (2016) "Identification and Quantification Analysis on the Chemical Constituents from Traditional Mongolian Medicine Flos Scabiosae Using UHPLC–DAD–Q-TOF-MS Combined with UHPLC–QqQ-MS," Journal of Chromatographic Science, 54(6), p. 1028. doi:10.1093/chromsci/bmw041.
79. Becht, A. et al. (2021) "Tracing the origin of paracetamol tablets by near-infrared, mid-infrared, and nuclear magnetic resonance spectroscopy using principal component analysis and linear discriminant analysis," Analytical and Bioanalytical Chemistry, 413(11), p. 3107. doi:10.1007/s00216-021-03249-z.
80. Zhao, M. et al. (2022) "Identification, synthesis and characterization of avanafil process impurities and determination by UPLC," RSC Advances, 12(15), p. 9256. doi:10.1039/d2ra01224c
81. Wu, S.S., Chein, C.-Y. and Wen, Y.-H. (2008) "Analysis of Ciprofloxacin by a Simple High-Performance Liquid Chromatography Method," Journal of Chromatographic Science, 46(6), p. 490. doi:10.1093/chromsci/46.6.490.
82. Liu, M. et al. (2007) "Extraction and ultra-performance liquid chromatography of hydrophilic and lipophilic bioactive components in a Chinese herb Radix Salviae Miltiorrhizae," Journal of Chromatography A, 1157, p. 51. doi:10.1016/j.chroma.2007.05.018.
83. Gorman, P.M. and Jiang, H. (2004) "Isolation methods I: Thin-layer chromatography," in Separation science and technology. Elsevier BV, p. 203. doi:10.1016/s0149-6395(03)80011-9.
84. Betz, J.M., Brown, P.N. and Roman, M. (2010) "Accuracy, precision, and reliability of chemical measurements in natural products research," Fitoterapia. Elsevier BV, p. 44. doi:10.1016/j.fitote.2010.09.011.
85. Singh, D.K. et al. (2018) "Pharmaceutical Analysis—Drug Purity Determination," in Elsevier eBooks. Elsevier BV. doi:10.1016/b978-0-12-409547-2.14551-2.
86. Madadlou, A., O'Sullivan, S. and Sheehan, D. (2010) "Fast Protein Liquid Chromatography," Methods in molecular biology, p. 439. doi:10.1007/978-1-60761-913-0_25.
87. Unger, K.K. et al. (2010) "Liquid Chromatography—Its Development and Key Role in Life Science Applications," Angewandte Chemie International Edition. Wiley, p. 2300. doi:10.1002/anie.200906976.
88. Rosen, R. (2007) "Mass spectrometry for monitoring micropollutants in water," Current Opinion in Biotechnology, 18(3), p. 246. doi:10.1016/j.copbio.2007.03.005.
89. Bhavyasri, K., Saileela, S. and Sumakanth, M. (2023) "high-resolution mass spectroscopy (HRMS)-a review," International Journal of Current Pharmaceutical Research, p. 9. doi:10.22159/ijcpr.2023v15i3.3008.
90. Sasidharan, S. et al. (2010) "Extraction, Isolation And Characterization Of Bioactive Compounds From Plants' Extracts," African Journal of Traditional

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Complementary and Alternative Medicines, 8(1). doi:10.4314/ajtcam.v8i1.60483.

91. Hage, D.S. (1999) "Affinity Chromatography: A Review of Clinical Applications," Clinical Chemistry. American Association for Clinical Chemistry, p. 593. doi:10.1093/clinchem/45.5.593.
92. Farouk, F., Moussa, B.A. and Azzazy, H.M.E. (2011) "Fourier transform infrared spectroscopy for in-process inspection, counterfeit detection and quality control of anti-diabetic drugs," Spectroscopy, 26, p. 297. doi:10.3233/spe-2011-0531.
93. Kim, E.J. et al. (2021) "Process Analytical Technology Tools for Monitoring Pharmaceutical Unit Operations: A Control Strategy for Continuous Process Verification," Pharmaceutics. Multidisciplinary Digital Publishing Institute, p. 919. doi:10.3390/pharmaceutics13060919.
94. Orlandini, S., Pinzauti, S. and Furlanetto, S. (2012) "Application of quality by design to the development of analytical separation methods," Analytical and Bioanalytical Chemistry, 405, p. 443. doi:10.1007/s00216-012-6302-2.
95. Xie, W. et al. (2019) "Interpretable Biomanufacturing Process Risk and Sensitivity Analyses for Quality-by-Design and Stability Control," arXiv (Cornell University) [Preprint]. doi:10.48550/arxiv.1909.04261.
96. Mohamed, M. et al. (2021) "Determination of microbial recovery for some pharmaceutical raw materials and passive monitoring at production area and microbiology laboratory at Egyptian pharmaceutical company," Journal of Advanced Pharmacy Research, p. 0. doi:10.21608/aprh.2021.72862.1127.
97. Prasad, S.K., Diwekar, K. and Anita.A (2020) "QBD, review on comprehensive understanding of building an analytical quality by design for drug manufacturing process," Journal of Biomedical and Pharmaceutical Research, 9(2). doi:10.32553/jbpr.v9i2.744.
98. Anderson, N.G. (2012) "Optimizing Processes by Minimizing Impurities," in Elsevier eBooks. Elsevier BV, p. 237. doi:10.1016/b978-0-12-386537-3.00009-5.