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Development techniques in column chromatography

Chromatography techniques play a crucial role in pharmaceutical analysis by identifying, separating, purifying, and quantitatively estimating complex mixtures of organic compounds. The development of analytical methods using chromatography is vital for pharmaceutical applications, particularly for assay and impurity profiling studies. A suitable method is established after evaluating critical separation parameters, such as stationary phase, column temperature, flow rate, solvent system, elution mode, and injection volume. Chromatography's significance in pharmaceutical drug development cannot be overstated, allowing for the precise estimation of targeted analytes in drugs. The technique has been pivotal in streamlining processes for organic chemists and industries alike. The use of various chromatography techniques, including thin-layer chromatography, paper chromatography, liquid chromatography (HPLC/UPLC), supercritical fluid chromatography, and gas chromatography, has expanded the capabilities of pharmaceutical analysis. This chapter focuses on the application of ultra-high-performance liquid chromatography (UPLC/HPLC) in pharmaceutical drug development, drawing from the work of Archer John Porter Martin and Richard Laurence Millington Synge, who won the 1952 Nobel Prize in Chemistry for their contributions to the field. HPLC and UPLC Techniques for Analytical Method Development in Pharmaceuticals In pharmaceutical industries, stability-indicating HPLC/UPLC methods are crucial to estimate assay and determine impurities of new drug substances and products. Assay is a quantitative test to determine individual components present in a substance, while impurity is an unknown component that affects the quality, safety, and efficacy of drug substances. To develop suitable chromatography methods, several parameters must be chosen for optimal separation and purity. These include: - Chromatography mode - Detector selection - Column (stationary phase) selection - Mobile phase buffer strength and pH - Organic modifiers - Ion-pair reagents - Flow rate - Solvent delivery system - Diluent selection Additionally, other critical factors such as forced degradation studies, mass balance study, stability of solution, and robustness of the method must be evaluated. Before starting an analytical method development, referring to literature on column characteristics for target molecules can aid in designing a method. The solubility profile of the drug substance helps select diluents for standard solutions and extraction solvents for test solutions. Analytical profiles consider physicochemical properties like pKa, melting point, degradation pathways, and absorption characteristics to select detector wavelengths. Stability profiles inform about storage conditions and handling precautions. Impurity profiles collect information on unknown components, which is essential in ensuring quality and safety standards. The process of developing a method for separating impurities and degradation products of a targeted analyte involves understanding the formation pathways of the drug substance. This information helps in identifying potential impurities and degradation products, which can vary depending on the manufacturing process. Metabolic pathways play a crucial role in determining possible degradation products, as they involve chemical reactions between the drug molecule and enzymes within cells. Understanding these pathways provides critical inputs on the possible degradation products. A stability-indicating method is essential for identifying closely related structures of molecules and impurities. This involves collecting structural information on the molecule and its impurities and degradation products, which helps in developing a specific and stability-indication method with good resolution between closely related structures. Functional group analysis, based on structural analysis techniques, helps in understanding the polarity of the drug molecule. By comparing the structures of impurities and degradation products with the structure of the drug molecule, scientists can determine polarity based on functional groups, making it easier to choose the right solvents for separation. Estimation of maximum daily dose (MDD) is crucial for calculating reporting, identification, and qualification thresholds of the drug substance and product. This information can be obtained from various sources, including ICH Q3A guidelines, physical desk references, innovator product information leaflets, and online databases like RX-list. Chromatography modes, such as normal and reverse phase, play a significant role in separating components based on their properties. The choice of mode depends on the type of sample being separated, with reversed-phase chromatography being the preferred mode for most molecules except isomers. Detectors, including UV-vis, refractive index, and evaporative light scattering detectors, are essential for finalizing analytical methods. These detectors help in quantifying and analyzing molecules and their associated impurities by detecting absorbance maxima or area intensity of test compounds using calibration curves. Chromatography can be employed for quantitative analysis of assay and impurities [11]. If compounds are non-chromophoric, using multiple detectors such as UV, RI/ELSD/CAD is recommended to capture all impurities. Alternatively, non-chromophoric compounds can be analyzed by UV after conversion into a derivative. However, the use of derivatives must be carefully evaluated considering the functional group involved [12, 13]. Fluorescence detectors (FLD) can be used for molecules with fluorescence properties, but their application requires consideration of available structural information for all possible impurities and degradation products [14]. The choice of column is crucial as it determines the success of chromatographic separations. Various columns are available, offering flexibility in changing parameters. Silica-based columns are commonly used, particularly in normal-phase chromatography with nonpolar organic solvents. The surface silanol groups provide a polar character to silica. Over time, silica support has been modified through three generations of manufacturing technologies. Type A and B silicas have improved properties by reducing metal impurities. Functionalized silica columns with varying levels of cross-linking and substitution of aliphatic and aromatic moieties can separate mediums of different polarities. When selecting a column, several parameters must be considered: column length and diameter, packing material, particle shape and size, carbon loading percentage, and internal bed dimensions. A shorter column (30–50 mm) may result in faster run times but lower resolution, while longer columns (250–300 mm) can provide higher-resolution separations. Decreasing particle size increases resolution, but also raises back pressure. Smaller particles are generally more efficient, yet might lead to high back pressure limiting separation efficiency. Typically, particles of 3 micrometers or less are used for complex and multicomponent samples, resulting in better resolution and separation characteristics due to lower surface area. Larger pores enable longer retention times through maximum surface area exposure for bigger solute molecules. Higher surface area provides greater retention, capacity, and resolution for multiple component samples, but low surface area materials equilibrate quickly, offering less efficient separation yet preferred in gradient analyses. Carbon loading typically offers higher resolution and longer run times with higher carbon loads, whereas lower loads shorten run times and alter selectivity. End capping reduces peak tailing of polar compounds that interact excessively with the column's exposed silanols. Non-end capped packing exhibits different selectivity, especially for polar compounds. Real separation occurs when the adsorbed compound is eluted using a mobile phase with the required polarity. Mobile-phase selection involves considering parameters such as buffer choice and pH, composition, and eluting efficiency. Organic/inorganic buffers like phosphate, acetate, triethylamine/diethylamine, and ion-pair reagents are used to reduce tailing factors and achieve well-separated peaks. The buffer's retention time is delayed when more concentrated, with optimal molarity ranging from 0.05 to 0.20 M. Buffer strength can be adjusted depending on the separation requirements, between 10% and 20%, but variations should be studied in detail to prevent precipitation or turbidity. Buffer selection and pH optimization play crucial roles in achieving effective chromatographic separations. The pH of the buffer or mobile phase significantly impacts elution properties by controlling ionization characteristics, which in turn affects retention times. The pKa value of the analyte or test mixture determines the optimal pH range for separation. A higher pKa requires a lower pH or acidic mobile phase to prevent unwanted association with the stationary phase. For acid-sensitive compounds, a low pH is necessary, while basic compounds require a high pH or basic mobile phase. Neutral compounds can be separated using neutral mobile phases. Maintaining the pH of the mobile phase within the 2.0-8.0 range is essential, as columns beyond this range can become deactivated due to siloxane linkages. To enhance separation efficiency, binary and tertiary solvent mixtures are often used in conjunction with buffers and acids or bases. The ratio of organic to aqueous solvents can be adjusted to achieve optimal selectivity. Experiments should be conducted with varying mobile phase compositions to determine the best separations between impurities. To separate analytes with similar properties, optimize for one component by adding an ion-pair reagent that modifies polarity. Carefully select a suitable reagent to achieve necessary selectivity. When employing an ion pair reagent (0.0005 M to 0.02 M), use a dedicated LC column and establish a cleaning procedure to extend the column's lifetime. Alkyl ammonium salts are useful for acidic compounds, while alkyl sulfonate salts work well for basic compounds. Sodium perchlorate can be used for acidic components. Separation is influenced by mobile phase flow rate inside the column. Optimize the flow rate based on retention time, back pressure, and separation of adjacent peaks or impurities. Preferably, fix the flow rate at no more than 2.0 mL/minute to achieve good peak symmetries and minimal back pressures. Temperature also needs optimization as it affects flow rate and adsorption rates. Choose a temperature that balances resolution between adjacent peaks and minimizes degradation of the test sample. Ambient temperatures are preferred, but higher temperatures (up to 60°C) can be used if necessary and confirmed stable for the compound. Chromatographic separations using single eluents are generally preferable, but gradient elution can achieve better separation between closely eluting compounds or those with narrow polarity differences. Prior to achieving better separation, perform experiments using different mobile-phase combinations and gradient programs. In a gradient run, premix two mobile phases with different polar and nonpolar solvent compositions using a single pump before introducing them to the column (low-pressure gradient). When setting up chromatography, it's crucial to choose the right solvent gradient system. The high pressure gradient (HPG) method is better when more than 80% organic phase is pumped, but it's essential to monitor system pressure and flow rate to avoid issues with low-viscosity solvents like acetonitrile. Optimizing the gradient program involves checking for carry-over after each run and ensuring the system stabilizes before the next injection. A standard method development starts with a 50:50 buffer and mobile phase, gradually increasing to 5:95 over at least 30 minutes, then reversing to 95:5. Diluents should be chosen based on solubility, extraction efficiency, peak shapes, and compatibility with the mobile phase. The diluent's impact on resolution of impurities and peak symmetry is also crucial. General extraction methods include sonication, rotary shaking, or a combination of both. To confirm the stability of a substance, heating can be tested if it remains stable and doesn't precipitate when cooled to room temperature. A mixture of impurities, starting materials, by-products, intermediates, and degradation products is used to establish separations. If all impurity samples are not available initially for method development, reaction mass/mother liquor/what if study samples are utilized. Forced degradation samples are prepared if degradation products aren't available in the case of drug API. A placebo solution with a concentration equivalent to the test concentration is prepared to check for interference from blank and placebo components. Individual solutions of standard and impurities are injected to confirm retention times, and a gradient program assesses elution patterns. An isocratic run is also conducted with a mobile phase of buffer and acetonitrile in a specific ratio using a silica column. The test concentration and injection volume are selected based on the API peak response at the detector wavelength. After finalizing the test concentration, a test solution is prepared, and its clarity and turbidity are checked after 24 hours. The method is considered stability-indicating if it meets the peak purity requirement. Forced degradation studies aim to investigate likely degradation products, establish degradation pathways, and ensure intrinsic stability of the drug molecule. Major forced degradation studies include stress testing due to high temperature exposure, which induces bond breakage and solid-state reactions following an autocatalytic pathway involving an induction period, rapidly increasing degradation, and slowing down as the compound is consumed. When plotting degradation vs. time, curves may form. Before conducting thermolytic degradation, determine the melting point of compounds. Then, choose a temperature based on melting points: below 100°C, use 70°C; between 100°C and 150°C, subtract 40°C from the melting point; above 150°C, stress at 105°C. Keep samples in an oven for one week or until 2-20% degradation is reached, whichever comes first. Stress drug substance, placebo, and drug product separately. For multicomponent products, test placebo with other actives, excluding one at a time. Hydrolytic degradation involves hydrolysis reactions, which are typically acid or base catalyzed. Employ acidic, neutral, and basic conditions to induce potential hydrolytic reactions. Estimate the solubility of drug molecules in water first; if not soluble, use a slurry or suspension or add a cosolvent like acetonitrile or methanol. Use caution when adding methanol under acidic conditions, especially with compounds containing carboxylic acids, esters, or amides. Perform hydrolytic stress testing at about 70°C with a reflux condenser to avoid evaporation. Stress until 2-20% degradation is reached. Neutralize stressed solutions before injection and prepare stressed solutions at higher concentrations than test concentrations. For multicomponent products, test placebo with other actives, excluding one at a time. Oxidative drug degradation reactions are typically autoxidative, initiated by radicals. Initiation phase followed by propagation and termination phases can lead to S-shaped curves in degradation vs. time plots, deviating from Arrhenius kinetics. Oxidative stress studies require temperatures below 30°C to avoid reduced reaction rates. Degradation should be performed at room temperature (25–30°C) with constant stirring in the dark, using 3% hydrogen peroxide. Stress testing should be conducted for 24 hours or until 1–20% degradation is achieved. For multicomponent drug products, separate stress testing of placebo and other actives excluding one at a time is recommended. Photolytic degradation involves exposure to UV or visible light, with samples stressed for 3 times 1.2 million lux-hr visible and 200 W-hr/m2 UVA. Peak purity can be evaluated for the main peak and major degradants, with degradation products identified through co-injection and comparable spectra. If known impurities increase in stress, proper examination should be conducted. Process impurities found in stress studies require assessment of secondary pathways of formation. After removing all contaminants and degradations, absorption spectra are recorded and compared by overlaying known impurities with the main analyte under each stress condition. A wavelength is selected where all impurities are detected and quantified, with maximum absorbance. If this is not feasible, alternative wavelengths are chosen to estimate impurities' presence. Lower wavelengths (210-220 nm) can be used to detect any additional impurities missing at higher wavelengths, which may occur when the parent compound breaks into two parts during stress testing, one part highly UV active and the other with poor UV character. The stability of analytical solutions is established by continuously injecting samples on an auto-injector for at least 12 hours in sequence mode to ensure the ruggedness of the method. System suitability tests verify the system's performance according to set criteria, focusing on critical separation parameters like resolution factor. Before finalizing these parameters, robustness studies are conducted to understand their behavior under various deliberate changes in method conditions. Two different HPLC systems must be used for system suitability checking when impurity separations are critical. Robustness studies involve testing mobile phase composition, pH, gradient, flow rate, and temperature variations to ensure the developed method is stability-indicating. The relative response factor is used to correct detector responses of impurities versus the main analyte peak, primarily controlling impurities or degradation products in drug substances or products. Liquid Chromatography Methods for API and Impurity Analysis ---------- The standard solutions of API and impurities can be prepared in various concentrations, ranging from 0.1% to 1.0%, and analyzed using liquid chromatography. The relative response factor (RRF) is calculated by comparing the slope of the impurity and the main drug (API). Several methods are available for quantitative determination of assay and organic impurities: * External standard method: This approach estimates impurities using a respective impurity standard without the API standard peak. * Area normalization: When the RRF value of known impurities is close to the API, this method can be used for quantification. Establishing recovery without response factors is necessary. * Diluted standard method: If the RRF values of impurities are different from the analyte, this method can be chosen. * Internal standard method: This approach is suitable when sample preparation procedures involve multiple extraction steps to avoid errors in the procedure. Chromatography methods development and optimization, especially for analytical techniques like HPLC, UPLC, and LC-MS, are crucial for separation and quantitative estimation of organic compounds. Liquid chromatographic methods are widely used for this purpose. This chapter focuses on major parameters controlling purification of organic compounds, including drugs, precursors, and degraded products. The following resources are available on various topics related to chromatography and analytical chemistry: * A textbook on metabolic pathways, "An Introduction to Metabolic Pathways" by S. Dagley (1971), provides an overview of biochemical processes. * Regulatory guidelines for impurities in new drug substances and products are outlined in the FDA's Q3A(R2) and Q3B(R2) guidelines (2008 and 2003, respectively). * Research articles on conformational analysis, spectroscopic determination, HPLC detectors, and liquid chromatography/post-column derivatization for amino acid analysis are available from various journals. * Reviews of derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry, fluorometric detectors, and the choice of buffers for reversed-phase HPLC are also provided. * Online resources on ZORBAX HPLC columns, the influence of mobile phase composition on retention factors, and the design of mobile phase composition for internal pH gradient chromatography are available. * Studies on the role of organic modifiers in RP-HPLC separation, the effect of organic modifiers on inclusion chromatography, and ion-pair reagents for HPLC provide additional insights. Note: I tried to paraphrase the text while maintaining its original meaning and context. The article discusses various aspects of high-performance liquid chromatography (HPLC), including selectivity, temperature, column thermostatting, sample preparation, and gradient separations. The first reference, Shibue et al. (2005), explores how the hydrophobicity of anionic ion-pairing reagents affects the selectivity of peptide separations by reversed-phase liquid chromatography. Another article (Good Habits for Successful Gradient Separations, 2019) provides guidance on gradient design and development to improve HPLC separation efficiency. Temperature and column thermostatting are also discussed in several references, including Heidorn's white paper (1999) and Thermo Scientific's poster note (2014). Sample preparation is a crucial step in HPLC analysis, as mentioned in articles by Slack et al. (2007) and LCGC Editors (2015), which provide an overview of sample preparation. Forced degradation studies are used to assess the stability of drugs and products, with Singh et al.'s article (2013) highlighting its importance. Additionally, references such as Dolan's article (LCGC North America, 2014) and Jain's article (Trends in Analytical Chemistry, 2013) offer insights into injecting mobile phases and forced degradation studies. The references also include articles from reputable sources like Waters, Agilent Technologies, and the International Pharmacopoeia, providing a comprehensive understanding of HPLC principles and applications. Recent advancements in analytical perspectives have significantly impacted the field of pharmaceutical and biomedical analysis, as highlighted by various studies published in reputable journals such as Journal of Pharmaceutical and Biomedical Analysis, Rasayan Journal of Chemistry, Pharmacopeial Forum, Chromatographia, and Química Nova.