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Review Paper

The Role of Stability-Indicating RP-HPLC In Ensuring Drug Product Quality and Shelf Life

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ABSTRACT

Reverse-phase high-performance liquid chromatography (RP-HPLC) is commonly employed in pharmaceutical analysis for the isolation and quantification of drugs, impurities, and degradation products. Ensuring the quality of drug products and the shelf life—determined by the potency, safety, and efficacy of a particular drug—is an important assessment that is carried out through stability studies, where environmental variables such as temperature, humidity, and storage conditions are tested to see how they affect the drug over time. Stability-indicating RP-HPLC method is one of the vital study since it provides accurate measurement of the decreasing drug content and precise estimation of the concentration of all degradation impurities. Method Development: This stage involves optimization of chromatographic conditions including mobile phase, the column, and detectors, among others, such that the separation and detection of analyte is optimal. To receive suitable and purpose-based validation according to ICH guidelines. RP-HPLC is widely used in stability studies to estimate the shelf life of drugs based on degradation rates over time. Despite being popularly used, RP-HPLC comes with many challenges—including sensitivity, matrix interference, method development, etc. Recent trends in RP-HPLC technology include advancements in columns, detectors and data processing software. Integration with other analytical techniques such as mass spectrometry has revolutionized stability testing and compound characterization. Stability testing has evolved with the introduction of automation and high-throughput screening, allowing for the fast, efficient, and comprehensive analysis of drug candidates and formulations.

INTRODUCTION

During RP-HPLC, incoming ions are injected into the chromatography column, with analytes being

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separated based on their hydrophobicity and retention factor (i.e., their degree of polar/non-polar character), providing analyses of high resolution, high sensitivity and good versatility, and therefore widely used in pharmaceutical analysis. This process is useful for separating and quantitating the drug and its impurities and degradation products (Jadhav et al., 2017). In RP-HPLC a nonpolar stationary phase and a polar mobile phase are used, enabling the separation of molecules based on their hydrophobicity. RP-HPLC has wide utility in pharmaceutical analysis, for quality control of bulk drug and formulations, assays of drugs in biological sample, and as a tool for degradation product determination (Yabré et al., 2018) When paired with different detectors like ultraviolet absorption or visible absorption in UV-Vis or photodiode arrays, or mass spectrometry, it is an incredibly powerful analytical technique (Fouda et al., 1991; Jadhav et al., 2017). For example, quality-by-design (QbD) approaches can be implemented to develop optimal methods for RP-HPLC systems to enhance the robustness and reliability (Shamim et al., 2023). Although RP-HPLC is commonly used in laboratories, the utilization of large portions of organic solvents poses environmental hazards associated with RP-HPLC. The development of greener RP-HPLC methods with alternative solvents or aqueous mobile phases are also underway (Yabré et al., 2018). Moreover, new technologies/hyphen such as AUltra-High-Performance Liquid Chromatography (UHPLC)/offer better resolution, sensitivity, and speed of analysis compared to conventional HPLC which can be used for pharmaceutical analysis (Cielecka-Piontek et al., 2013).

Introduction to stability studies and their importance in ensuring the quality and shelf life of drug products

This information is vital to quality assessment of drug products, and contributes to shelf life estimation, which is the purpose of stability studies. These investigations were performing studies exploring degradation, showing how environmental conditions, like temperature, humidity, and storage have an impact over time on the efficacy, potency and safety of a drug (Collier et al., 2010; Lyon et al., 2006). The significance of stability testing is that through it, the shelf life of a product can be accurately predicted, a crucial aspect for maintaining therapeutic efficacy throughout the storage duration (Kerr et al., 2019; Waterman, 2011). In fact, recent data generated by the shelf-life extension program (SLEP) indicate many drug products remain stable well beyond their labelled expiration dates and even when not stored properly (Lyon et al., 2006). There was, however, considerable lot-to-lot variability which highlights a need for systematic evaluation. Advanced kinetic modeling (AKM) is another approach that has proven to be informative for long-term stability predictions using the finite accelerated stability data, enabling rapid shelf-life predictions that cohere with dictated requirements (Evers et al., 2022; Huelsmeyer et al., 2023; Waterman, 2011). In conclusion, stability studies are an inalienable part of pharmaceutical development ensuring product and formulation optimizations. It is critical for the selection of excipients, packaging materials, and storage conditions to limit degradation and ensure activity during the entire shelf life of the product (Collier et al., 2010; Medarević et al., 2019). These methods make more accurate and efficient stability predictions possible through the application of structural information and accurate accounting of crystallinity considerations, thus enabling enhanced quality control and regulatory compliance in the pharmaceutical industry.

Need for Stability-Indicating Methods

Stability-indicating methods are crucial in pharmaceutical analysis since the assessment of drug quality, safety, and efficacy during shelf life of a specific product is imperative. They aim to accurately measure variations in drug



concentration over time and reliably establish the levels of degradation impurities (Chew et al., 2021). These methods are necessary as unstable drugs may undergo changes in color, physical characteristics and effectiveness of the drug and even lead to toxicity (Dhondale et al., 2023). There are numerous challenges to the development of stability indicating methods. A major challenge is that high sensitivity is required to detect low levels of impurity. As one example, nitrosamine, which are highly potent carcinogenic impurities, must be detected with methods that have quantitation limits of < 0.25 ppb (Zheng et al., 2022). Another is handling complex matrices and a large number of samples needing analysis (Zheng et al., 2022). Moreover, new drugs, especially those for COVID-19, must be analyzed for multiple parameters of analytical methods using time-consuming techniques such as Quality by Design (Dongala et al., 2020) For numerous reasons, there is significant require for dependable and delicate techniques to identify degradation items. They assist in understanding the stability profile of drugs, as shown here for ibuprofen and phenylephrine (Kelani et al., 2023). These approaches also provide the means to identify and structurally elucidate impurities that are vital to ensure patient safety and a continuous drug supply (Maggio et al., 2014; Zheng et al., 2022). In addition, they enable rational decision making about impurity identity and level that should be produced in order to deliver a better and safer medicine (Maggio et al., 2014).

Purpose and Scope of the Review

RP-HPLC is a major analytical tool applied to stability testing of pharmaceutical candidates. The approach was particularly useful in the assessment of drug degradation products and related substances (Jadhav et al., 2017) during the analysis of linagliptin (LGP) and metformin HCl (MET HCl) tablets. The RP-HPLC method developed in this work was used for the separation of nine specified impurities at their qualified limit and validated as per ICH guideline, which was found as accurate, precise, reproducible, robust, and specific.

RP-HPLC creates exciting prospects for coupling to other analytical technologies for a comprehensive stability profile. With LGP, the same methodology was adapted for LC-MS and UPLC-TOF/MS for identification and characterization of degradation products during stability studies (Jadhav et al., 2017). This versatility allows for greater exploration of drug degradation pathways and tuning of performance over product lifetime. Essential Analytical Tool for RP-HPLC Analysis It is a significant method to make sure that the drug product remains effective and safe over its life cycle because it helps in the immobilization and quantitation of drug-related impurities and degradation products and also has the potential to be adapted with other analytical methods. The use of green RP-HPLC methods (Yabré et al., 2018), the environmental impact of stability testing can also be reduced, thus bringing more safety to analysts.

2. Fundamentals of RP-HPLC

RP-HPLC Principles

Reverse phase chromatography (RPC): RPC is a widely used separation method in analytical chemistry which utilizes a nonpolar stationary phase and polar mobile phase (Axente et al., 2023). The stationary phase is typically silica-based materials with chemically bonded octadecylsilyl (C18) or any hydrophobic groups (Black & Coon, 1982). This method allows separation of molecules based on hydrophobicity. For reverse phase chromatography, the mobile phase consists of a mixture of water and organic solvent, e.g. acetonitrile or methanol (Axente et al., 2023; Subirats et al., 2019). Depending on the column used, the composition of the mobile phase can be changed to help separation, a task that includes the use of additives such as buffers or ionic liquids to improve chromatographic separation (Axente et al., 2023). Ionic liquids are a promising approach as mobile phase modifiers in separating polar basic molecules, including nicotine and cotinine (Axente et al., 2023) Hydrophilic interaction liquid chromatography (HILIC) operates on a similar principle, except



that it employs a polar stationary phase and a less polar mobile phase, consisting mostly of a high volume fraction of organic solvents (Buszewski & Noga, 2011; Subirats et al., 2019). The separation of polar analytes that are challenging or impossible to analyze by reverse-phase is also becoming increasingly performed by this method (Dias et al., 2021). Like HILIC and reverse-phase chromatography, these techniques can have completely different retention mechanisms (Subirats et al., 2019), allowing complementary selectivity and the comprehensive analysis of complex samples.

Types of detectors commonly used in RP-HPLC

Several types of detectors are applied in RP-HPLC but among the most popular are UV-Vis and fluorescence detectors. UV-Vis detectors are commonly used in RP-HPLC due to their general applicability. They work well for compounds that absorb light in the ultraviolet or visible regions of the spectrum. UV detection at 270 nm have been used for abscisic acid analysis in barley (Nakurte et al., 2012), and with diode-array detectors monitoring synthetic food dyes in vitamins in the range between 190 and 800 nm (Šuleková et al., 2016). Compounds like mycophenolic acid have also used UV detection at 304 nm (Danafar & Hamidi, 2015). Fluorescence The detectors are sensitive and selective for fluorescent species or for species that can be derivatized and made fluorescent. They are especially useful for trace analysis. The method has found application in the detection of indole-3-acetic acid and indole-3-pyruvic acid in cereal grains (e.g., barley) with detection at excitation wavelength 282 nm and emission wavelength 360 nm (Nakurte et al., 2012), and also for polycyclic aromatic hydrocarbons in water samples (Bortolato & Olivieri, 2014) This method based on the labelling of sulfenic acids with monobromobimane and subsequent RP-HPLC and fluorescence detection solved the problems above and reached a low detection limit of 2 nM for sulfide dibimane (Shen et al., 2015). In fact, there is even evidence that several studies have been conducted where more than one detection method was employed [47,

48]. An example is a method for determining polycyclic aromatic hydrocarbons in food supplements using UV and fluorescence (Danyi et al., 2008). Another type of detectors like evaporative light scattering (ELS) has also been used in some studies for special purposes, such as the analysis of steroidal saponin in *Tribulus terrestris* (Ganzera et al., 2001). The selection of detector for RP-HPLC relies on the analytes and sensitivity needed. UV-Vis and fluorescence detectors are very common detectors due to their versatility and sensitivity, respectively, but using multiple detection methods or specialized detectors can provide comprehensive analytical solutions for complex samples.

Advantages of RP-HPLC

The advantages of HPLC in RP-mode are as follows: high resolution, sensitivity, reproducibility, and versatility, with applicability to a broad range of compounds. 8 RP-HPLC, a high resolution and sensitive technique 810. This was particularly effective in the sugar analyses in mushrooms by the HPLC technique, in combination with a corona-charged aerosol detector (HPLC-CAD) showing good linearities and detection limits (Sławińska et al., 2020). Similarly, the UPLC-ESI-MS/MS amino analysis method was able to detect as little as one atomole, possessing one to five orders of magnitude higher sensitivity than existing methods (Salazar et al., 2012). The RP-HPLC method is versatile, with compound classes from sugars and amino acids to mercury species and polyphenols that can be analyzed. In addition, HS-SPME-RGD have also been applied for quantification of target analytes, a current summary for quantification applications demonstrate simultaneous separation of nine purine and pyrimidine bases (Markelj et al., 2016), complex mixture of sugars following derivatization (Vojvodić Cebin et al., 2022) and four monosaccharides in *Osmanthus fragrans* Lour (Fan et al., 2018). Keys show very high reproducibility for a variety of applications by RSD < 5% in most cases (Faraji & Adeli, 2016; Sławińska et al., 2020). In conclusion, RP-HPLC is a valuable analytical technique characterized by



its high resolution, sensitivity, reproducibility, and versatility, enabling its application in a range of fields such as food science, metabolomics, pharmaceutical analysis, and many others. This emphasizes its importance in modern analytical chemistry, as they can manipulate complex matrices and provide reliable reproducible answers.

3. Stability-Indicating Methods in Pharmaceutical Analysis

Definition and Importance of Stability-Indicating Methods

An assay of stability is an analytical approach employed to monitor the concentrations and degradation products of drug substances in various formulations over time, ensuring the quality of pharmaceutical products and their safety (Chew et al., 2021). These techniques are critical during the testing of pharmaceuticals since they allow accurate estimations of the quantity of drug substance present and the levels of potential impurities, both of which must be carefully maintained for drugs to remain effective and safe for the duration of their shelf-life (Chew et al., 2021; González-González et al., 2022). Regulatory Agencies like FDA and ICH mentions about COA's (Clinical outcome assessment) and it surly proves the method stability indicating methods are of extremely importance. To standardize stability testing within the European Union, Japan, and the USA, ICH issued guidelines (ICH Q1A-E, Q3A-B, Q5C, Q6A-B) so that stability-testing practices within these 3 major markets would be consistent (González-González et al., 2022). In these guidelines, unique storage conditions and testing durations are defined for long-term, intermediate and accelerated stability studies which allow for an extensive assessment of the stability of the drug (González-González et al. 2022). While traditional ICH stability studies are both extensive and time-consuming, modern methodologies like Accelerated Predictive Stability (APS) studies are becoming more prevalent. The research took place over 3-4 weeks under extreme conditions with the aim of better predicting long-term stability

(González-González et al., 2022). Moreover, the FDA also has accepted new drug applications with regulatory flexibility for quality by design (QbD)-based analytical approaches, which emphasizes on the Analytical Quality by Design (AQbD) that can minimize out-of-trend and out-of-specification results due to enhanced method robustness (Peraman et al., 2015). In short, stability-indicating methods are of utmost importance to ensure pharmaceutical quality and safety; they must align with, or go beyond, the needs of regulation, and are also extremely useful when it comes to characterizing drug substance behaviour over time. So, they are only being enhanced over so that evolving and new techniques to make the drug testing process fluid.

Key Characteristics of Stability-Indicating Methods

Stability-indicating assays are critical for enabling stability studies of drug substances and pharmaceutical products. There are some key characteristics that make these methods reliable and efficient. stability-indicating assay must fulfill four critical parameters, such as selectivity, sensitivity, specificity, and reproducibility (Chew et al., 2021). In order for these methods to be able to accurately measure the changes in drug substance concentrations over time and quantitatively estimate the amount of degradation impurities (Chew et al., 2021), they need to be adequately validated per ICH guidelines. These assays must allow for the separation and recovery of the drug substance from impurities (Chew et al., 2021). The traditional approach for stability-indicating assays depends on chromatographic techniques (e.g. HPLC, GC, HPTLC), but the advent of hyphenated systems, in which chromatographic separation is coupled with spectroscopic detection, has resulted in stability studies being carried out using these advanced methods, which have been utilised in a two-dimensional manner. These systems, including HPLC-DAD, GC-MS, and LC-MS, facilitate simultaneous quantitative and qualitative dosing of drug substances and impurities (Chew et al., 2021). This improves the method's capability to



identify and characterize degradation products. In the end, the stability-indicating methods should be proved to be very sensitive, specific, accurate, and reproducible at the best precise drug quantity and lasers. Hyphenated systems are created by combining chromatographic and spectroscopic techniques, beneficial because they offer expanded capabilities of impurity characterization and identification, enabling robust stability-indicating methods (Chew et al., 2021).

4. RP-HPLC in Stability Studies (2 pages)

Role of RP-HPLC in Drug Stability Testing

The development of RP-HPLC for stability testing on drugs based on the separation, identification and quantitative assessment of the active pharmaceutical ingredient (API) and its degradation products. Its usability has been optimized mostly in terms of stability, degradation, and drug impurity analysis (Gupta et al., 2022; Jadhav et al., 2017). Hence, RP-HPLC would be an adequate method to develop stability-indicating methods for the separation and quantification of the API from its related substances and its degradation products. For example, Jadhav et al. To produce a new RP-HPLC novel method for linagliptin and its related substances determination in tablets seven impurities were resolved by one separation [3]. It was shown to be accurate, precise, reproducible, robust and specific for stability study and was validated as described by the ICH guidelines. RP-HPLC coupled with other analytical methods can provide more comprehensive information about the drug degradation. For example, the same RP-HPLC method for linagliptin was also suited for LC-MS and UPLC-TOF/MS to get m/z and fragmentation of unknown degradation products formed in the stability studies (Jadhav et al., 2017). The complementary nature of these techniques provides insightful information on degradation pathways of drugs, as well as information about unknown impurities. RP-HPLC is sensitive, specific, and reproducible and is a valuable technique in the stability testing of drugs. It enables pharmaceutical companies to study drug

degradation and impurities, detect and quantify these impurities, and assure stability of drug products over shelf-life. The versatility of RP-HPLC is further complemented with insight into drug degradation mechanisms and product quality characterization (i.e. during stability) when forming a hybridization with other analytical techniques.

Common types of stability testing

Forced degradation and shelf-life studies are the two primary forms of studies that are conducted for stability testing of pharmaceutical products (Bhangare et al., 2022; Evers et al., 2022). Forced degradation studies (known as stress testing) are experiments in which drug products are subjected to extreme conditions (e.g., high temperature, humidity, oxidation, and pH) to induce degradation as reported (Kerr et al., 2019; Muniandy et al., 2023). This will guide the investigation on possible pathways of degradation, and products of degradation. An example of this would be the application of hydrogen-deuterium exchange-mass spectrometry and collision-induced unfolding ion mobility-mass spectrometry techniques to study monoclonal antibody structure changes under accelerated storage conditions (Kerr et al., 2019). For this purpose, shelf-life studies are performed to assess the long-term stability of drugs when stored under the conditions recommended by the drug manufacturer (Evers et al., 2022; Lyon et al., 2006). Accelerated stability testing is often utilized to provide predictions (in a shorter time frame) of shelf life. One of these programs was the shelf-life extension program (SLEP) that evaluated the potency of drug products far beyond their labeled expiration dates and found that 88 percent of lots could be extended an average of 66 months (Lyon et al., 2006). Forced degradation and shelf life studies, thus, play a relevant role in the assessment of drug stability. Although information on degradation mechanisms can be gained through forced degradation, shelf-life studies are essential to define appropriate storage conditions and for development of expiration dates. State-of-the-art methods such as kinetic modelling can improve



the accuracy and efficiency of stability predictions for these systems (Evers et al., 2022; Waterman, 2011).

Case Studies of RP-HPLC in Stability Analysis

The stability studies of various pharmaceutical dosage forms have been carried out successfully using RP-HPLC. In this study we have developed and validated a RP-HPLC method for ointment formulations of Ozenoxacin and Benzoic Acid on C8 column using gradient elution. The procedure was simple, rapid, and suitable for routine QC testing. Also, forced degradation studies were performed to evaluate this method as a stability-indicating (Ramireddy & Behara, 2023). A stability-indicating RP-HPLC method has been developed and validated for simultaneous estimation of atorvastatin and amlodipine in combination drug products. Under the influence of thermal, photolytic, hydrolytic, and oxidative stress, the drugs and their degradation products were successfully separated from one another on a C18 column. In vitro dissolution studies for marketed combination products have also been performed using this method (Chaudhari et al., 2007). New RP-HPLC method being developed for quantitative determination of linagliptin as well as its excipients present in tablets form with metformin HCl. The method uses a design of experiments approach to maintain its robustness. Nine particular impurities were isolated and characterised as suitable marker compounds for bumps and peaks observed in the stability studies. For LC-MS analysis, slightly different approach was applied to identify the unknown degradation products (Jadhav et al., 2017). Rosuvastatin calcium was studied by the green RP-HPLC-UV method. The method was found to be stable, robust and able to separate rosuvastatin from its degraded forms. At present, it has been successfully used to study rosuvastatin, and self-nanoemulsifying drug delivery system, and OTCs (Haq et al., 2017). In summary, these RE-PAR;H studies illustrated the broad range and applicability of RP-HPLC as a reliable tool to perform stability-indicating assays for both single-drug formulations as well as combination products and

to separate at the same time identify even both specified and unspecified degradation products.

Impact of Environmental Factors on Stability

Changes in the stability of a drug due to environmental factors, especially light, temperature, humidity, and atmosphere, can be monitored using reversed-phase high-performance liquid chromatography (RP-HPLC). The primary environmental factors that impact drug stability are temperature and humidity. For example, the active pharmaceutical ingredient (API) levothyroxine demonstrates stability under numerous temperature and humidity conditions as well as within a potency range of 90%-110% (Collier et al. However, in the presence of moisture and high temperatures, some excipients, like croscopovidone, povidone, and sodium laurel sulfate are known to cause substantial degradation of the API by deiodination and deamination (Collier et al., 2010) A similar behavior was observed for caffeoylquinic acids (CQAs), which were degraded or isomerized when subjected to temperature, pH, and light conditions (Xue et al., 2016). Drug stability was also strongly influenced by light exposure. For example, for CsPbI₃, exposure to an above-bandgap laser in a humid environment forced the high-temperature phase into its low-temperature phase at rates substantially greater than that of the moisture-promoted phase transformation alone (Lin et al., 2022). It illustrates the interdependencies of light exposure and moisture, which influence the stability of materials. Stability in drug refers that the storage conditions are very important. Another area that can impact module long term performance is the storage of uncured encapsulant rolls used in photovoltaic modules. UV exposure, simulating 10 years of outdoor service, resulted in different levels of chemical and physical degradation in modules containing ethylene vinyl acetate (EVA) copolymers stored at different moisture levels (Gnocchi et al., 2023). RP-HPLC is known to be an important technique to monitor changes due to environmental factors in the stability of drug substances. This data has been used to evaluate the efficacy of maintaining CQAs



under diverse storage conditions (Xue et al., 2016), to develop a stability-indicating method for drugs such as linagliptin (Jadhav et al., 2017), and to quantify crocins in commercial liquid saffron extracts subjected to environmental factors (Suchareau et al., 2021). The detection of degradation products using RP-HPLC coupled with mass spectrometry provides information on the degradation pathways/mechanisms (Jadhav et al., 2017). The stability of drugs is greatly influenced by environmental factors. RP-HPLC is thus frequently combined with other analytical techniques and is a known method to monitor and understand these changes, providing insight into developing more stable formulations and optimal storage conditions for pharmaceutical products.

5. Method Development for Stability-Indicating RP-HPLC

Extensively, stability determining RP-HPLC methods are in use for pharmaceutical drugs and their degradation products. Several approaches have been developed to this end for these types of methods to maximize the robustness and reliability thereof. Several studies have employed quality-by-design (QbD) for method development. For example, applying QbD approaches, Dongala et al. (2020) developed a stability-indicating HPLC method for the impurities in hydroxychloroquine sulfate, such that several analytical parameters were assessed with a minimal number of experiments. Likewise, a methodology was successfully outlined for canagliflozin analysis by HPLC in accordance with the AQbD guidelines, where by factorial experimental design were utilized to optimize the pertinent parameters (Azhakesan & Kuppasamy, 2023). Patterned on green chemistry principles, many studies concerning the design of RP-HPLC methods have also been recorded. Different approaches have been suggested to reduce the ecological footprint of HPLC methods, such as replacing traditional organic solvents with alternative greener aqueous solvents (sacrificing less polar compounds from analysis), using fully aqueous mobile phases, or using micellar liquid chromatography (Yabré et al., 2018). This idea aims to minimize the use of

toxic solvents while ensuring similar analytical performance. In summary, systematic approaches like quality by design, though there is room for improvement in pattern emerging as known seems to be driving the development of stability-indicating RP-HPLC methods, exploring new avenues can potentially lead to improved critical method parameters and important traits as well. In the same line, we have slowly headed to greener practices thus trends will follow the same with pharmaceutical processes and analysis.

Selection of Chromatographic Conditions

Consider the Chromatographic conditions on Analyte separation and detection of all HPLC methods Selecting the mobile phase, column et al. mobile phase conditions; mobile phase composition, pH, solvent selection controlled the separation efficiency. For example, (Ventouri et al., 2023), explored aqueous mobile phases containing volatile salts at physiological pH for size-exclusion chromatography coupled with native mass spectrometry (SEC-nMS) while (Jovanović et al., 2015) performed HILIC using acetonitrile and water as the mobile phase containing ammonium acetate buffer solution (pH 6.5). For example, an optimal mobile phase pH of 5.7 have been reported for simultaneous quantum of 19 free amino acids in tea samples (Li et al., 2018). Also critical are column selection, flow rate and temperature. (Ventouri et al., 2023) employed 15- μ L/min flow rates using narrow SEC columns (1.0mm i.d.) that led to exquisite protein-ionization performance. The separation of 16 compounds from *Artemisia ordosica* was conducted using an Agilent Eclipse Plus C18 column (250 mm \times 4.6 mm, 5 μ m) with a flow rate of 1.0 mL/min and the column temperature at 40 °C (Kang et al., 2023). They used a GoatOn HILIC Analytical Column at subzero temperatures, (-30 °C), along with faster analytical flow rates and reduced back pressure than traditional RVPC (Anderson & Hudgens, 2023). In summary, the choice of chromatographic conditions is highly dependent on the analytes of interest and the objective of the analysis. The various parameters of mobile phase composition



(such as pH), column type (together with dimensions and type of stationary phase), flow rate and temperature must be optimized to generate the best separation and detection performance. In such cases, experimental design methods 14, are useful to explore space to systematically 15 optimize these parameters 16 and ensure a well-tuned method robust 17 performance.

Optimization of RP-HPLC Methods

High-performance liquid chromatography (HPLC) is considered a strong methodology for the separation and detection of degradants. To attain maximum resolution and detection sensitivity, there are various RP-HPLC methods optimization strategies. Optimizing HPLC Methods by Choosing the Right Mobile Phase Composition As an example, with minor modifications, the use of a methanol/water (8/2) mobile phase was employed to quantify a renin inhibitor with very low detection limits (Fouda et al., 1991). The optimization of separation and sensitivity can be largely affected by sampling ratios of the organic solvent to water Column selection is one of the major features. Reverse-phase columns are typically employed but the specific type would depend on the targeted analyte. Liu et al. (2005), although it was developed using HPLC with UV detection on a diode array detector, it showed how important it is to choose the right detection mode alongside the appropriate column (Liu et al, 2005). The detection method may contain enhanced sensitivity such as HPLC and mass spectrometry. MariN and Barbas (2004) coupled LC to MS to analyze degraded samples of cold cough products and obtain molecular weight information (MariN & Barbas, 2004). These new methods also help to identify unknown degradation products. In summary, RP-HPLC must be optimized for the resolution of degradation products, diluting them as much as possible while keeping their peaks in the linear range of detection. The coupling of HPLC and mass spectrometry further gives structural information, increasing the overall analytical power of the technique. These field conditions should be considered by researchers

when developing methods for specific applications, as exemplified in the articles cited.

6. Validation of Stability-Indicating RP-HPLC Methods (1 page)

Regulatory Guidelines for Validation

The International Conference on Harmonization (ICH) and other regulatory guidelines specify the requirements for validation of stability-indicating RP-HPLC methods. Such guidelines include ICH Q2(R2) which provides a holistic methodology for the validation of analytical methods (Chiarentin et al., 2023; Jadhav et al., 2017) and ensures that such methods are rugged, robust and exactly what the scientists intended to use. Stability indicating methods play a crucial role in the ICH Q1A-E regulations, which establishes the specific stability tests required for new drug entities and drug products. These are, typically, storage conditions, frequencies of tests, and days of studies needed to check (evaluate) a drug thermal stability and moisture sensitivity (González-González et al., 2022). Stability indicating methods are critical for ensuring the quality of new drug substances and products, and the recommendations for how to most appropriately achieve this for impurities are provided in ICH Q3A-B. Old way: Traditional ICH stability studies are exacting, and take a long time; new ones such as Accelerated Predictive Stability (APS) studies are gaining popularity. These 4 (extreme conditions)-week (or 3 (intermediate conditions)-week) studies result in a more rapid forecasting of long-term stability (González-González et al., 2022) for biopharma candidates. It shows how stability-testing approaches in the regulatory sphere continue to develop over time. Summary: This paper presents general guideline on stability indicating RP-HPLC method validation issued by ICH and the key determinations. These recommendations provide a reasonable foundation for implementing a general paradigm for method validation including key aspects of validation that include linearity, precision, accuracy, specificity, and robustness (Chiarentin et al., 2023; Jadhav et al., 2017;



Kowalska et al., 2022) It is likely that refinement of analytical methods will inform future regulatory guidance that balances the level of validation against the burden of not performing extensive testing to demonstrate product quality.

Key Validation Parameters

Among other things, published literature lists the various analytical methods, which address capture of these important validation parameters accuracy, precision, specificity, linearity, range, robustness, and detection limit. By means of the recovery study and RSD determination, it has often been estimated. Rahmani et al. (2010) mycotoxins were identified with intra- and interday precision and accuracy within the acceptable range, and recovery values varied from 77 to 104% depending on the concentration of mycotoxins in cereal samples (Rahmani et al., 2010). Moreover, a satisfactory recovery of N-nitrosamines in biopharmaceuticals (82.4%–116.8%) was similarly attained (Xie et al., 2023). Selectivity and specificity are generally evaluated by assessing the interference of sample matrices or related compounds. The selectivity and specificity of LC-MS/MS for detecting oregano adulterants have been also described (Wielogorska et al., 2017). Zusammenfassung (7) Auch die Multiparameter-Flowzytometrie-Nachweismethode von Tettero et al. (7) erwies sich als in der Lage, die häufigsten Leukämie-assoziierte Immunphenotypen mit ausreichender Sensitivität von negativen Kontrollzellen zu unterscheiden (Tettero et al., 2023). Calibration curves and correlation coefficients are commonly used to assess linearity. Other studies have high linearity reports ($R^2 > 0.99$ over a wide range of concentrations) (Jha et al., 2020, Kharat et al., 2016, Wielogorska et al., 2017). The main range is usually defined with linearity, and many papers report large working ranges that are appropriate for their target applications. Robustness was evaluated through intentional variation in the method parameters to produce consistent results. Not all studies outright test for robustness; those that do (e.g., (Alquadeib, 2018; Nakurte et al., 2012) validate that their methods are unaffected by minor changes in

system settings. Threshold levels such as the Limit of Detection (LOD) and Limit of Quantification (LOQ) are vital in trace analysis. Sensitivity was varied, and some methods have reported detection limits as low as femtomolar concentrations. The (Xie et al., 2023) (Xie et al., 2023) found LODs as low as 0.0037 ng/g for some aflatoxins ((Rahmani et al., 2010) reporteds LODs as low as 0.0037 ng/g for certain aflatoxins (Rahmani et al., 2010). Overall, these validation parameters are generally shared in all the methods including HPLC, LC-MS/MS and flow cytometry, as well as strictly follow ICH and FDA guideline criteria.

Challenges in Validation

Analytical processes including the sensitivity, accuracy, and reliability of separation techniques are greatly impacted as one of the substances in the sample matrix may interfere, known as matrix effects. Such effects may cause ion suppression/enhancement phenomena or modify the analyte signals at all stages of the analytical workflow (Williams et al., 2023). Every complex matrix is different and subject to different dynamic forces, which means that a pragmatic approach is needed for the analysis of such complex matrices that is also influenced by factors like the target analyte, sample preparation protocol, composition, and choice of instrument (Williams et al., 2023). Interestingly, Inactive ingredients that are otherwise non-active components known as excipients, in general, can significantly impact the performance of the drug products. Excipient critical material attributes or amounts may vary, posing risks for oral drug performance (Zarmpi et al., 2016; Zarmpi et al., 2020). For example, because magnesium stearate is lipophilic, the flowability of the drug can be lower, especially if the compound is highly soluble and/or highly ionized (Zarmpi et al., 2020). In contrast, superdisintegrants, while having a relatively low risk for impacting oral drug performance solely based on drug solubility, may still influence oral bioavailability through their effects on tablet disintegration (Zarmpi, Meehan, et al., 2020). This framework allows for identifying trends and analysing the cause of variability, both within a



given formulation and compared between different excipients, to ultimately lead to improved analytical development and better drug products.” To address these issues, better extraction and clean-up protocols have been proposed, separation using chromatographic techniques should be optimized, and calibration correction methods used (Williams et al., 2023). Also, it has been highlighted that the biopharmaceutical factors influencing excipient performance should be appreciated to successfully translate quality-by-design principles into pharmaceutical development (Zarmpi et al., 2016; Zarmpi et al., 2020).

7. RP-HPLC in Long-Term Stability Studies

Shelf-Life Prediction Using RP-HPLC

RP-HPLC has established a place in pharmaceutical analysis for quality control of bulk drugs and formulations and in its metabolic studies of drugs in biological samples (Yabré et al., 2018). This can also be applied to the development of stability-indicating—in this case an important aspect of shelf life prediction. For instance, a RP-HPLC method was established for concurrent estimation atorvastatin and amlodipine and the drug has been stressed under several conditions to obtain degradation profiles (web link, 2007). This is important to understand how stability of drug products with respect to different environmental conditions. Encompass drug degradation kinetics and the shelf-life as indicators, based on the obtained KPIs from the non-isothermal thermogravimetric and RP-HPLC studies.” Therefore, this strategy has been applied even to paracetamol formulations (Calvino et al., 2021) where cover up the variable storage conditions to estimate a shelf life range, since is well known that in the particular case of pharmaceutical products the expiration date is highly related to excipients composition. RP-HPLC has been proven to be a proper approach to evaluate the expiration date of the pharmaceutical drug product, based on its degradation rates. This allows us to create stability indicating methods, determine degradation products and investigate

the stability of drugs in different conditions. RP-HPLC, as a centrality tool, also in combination with other analytical analysis provides multi-dimensional information which supports accurate shelf-life estimation to maintain the potency of pharmaceutical products during their designed shelf-existence.

Analysis of Drug Products Over Extended Periods

When developing drug products, it is critically important to perform long-term stability studies to determine the degradation profile of that drug in the products being tested so that the potency of those products can be assessed for use throughout the product’s shelf life. These studies involve long-term stability investigations of the API and its degradation products at several storage conditions (Krake et al., 2023; Reichard et al., 2023). Solvent-free forced mechanochemical degradation can be applied in modelling long-term drug product degradation profiles excluding solvent-induced pathways, and irrelevant solution-state degradation pathways of degradation (Krake et al., 2023). Such an approach has been promising for predicting theoretical degradation profiles for novel drugs in drug formulations, by investigating a drug product comprising platelet inhibitors thienopyridine (Krake et al. 2023). In fact, the spaceflight environment has special problems for drug stability. Research has demonstrated that pharmaceutical products stored in low Earth orbit (LEO) show a slight increased rate of API loss compared to terrestrial controls, along with a correlated increase in the likelihood of product failure (Du et al., 2011; Reichard et al., 2023). Besides, spaceflight-exposed drugs have shown that their potency is normally still within 10% compared with terrestrial lot-matched controls, and only a ~1.5 increase in the degradation rate (Reichard et al., 2023). To summarize, long-term stability studies are necessary to assess degradation profiles of the drug and for predicting the effectiveness of the drug with time in the part of its assumed usage. Methods such as forced mechanochemical degradation and studies of various drugs in



spaceflight have offered key insights into the stability of drugs in regard to various conditions. Such studies will assist in helping develop more stable formulations and appropriate storage conditions for pharmaceuticals (Bhangare et al., 2022; González-González et al., 2022).

Example Studies and Data Interpretation

Numerous studies have explored the ability of RP-HPLC to predict or prolong the shelf-life of pharmaceuticals and food products. An example is developing a stability indicating RP-HPLC method for determination of linagliptin and its impurities in linagliptin/metformin HCl tablets (Jadhav et al., 2017). The method was validated according to the ICH guidelines which was found to be specific, accurate and precise for an effective online chromatographic separation for the quantitation of the drugs. The degradation of linagliptin was visibly higher at either basic or oxidative conditions with heat and humidity. By identifying and quantifying degradation products, this technique allows for the prediction of the stability and shelf life of pharmaceutical formulations. While RP-HPLC was also employed in the food industry in order to extend the shelf life of pasteurized milk (El Dessouky Abdel-Aziz et al., 2020). The aim of this study was to evaluate the influence of fig leaf extract (FLE), olive leaf extract (OLE), and mixed leaf extract (MLE) supplementation. RP-HPLC was used to identify these phenolic-compounds. The inclusion of 0.6% FLE, OLE, or MLE significantly inhibited the lipase and protease when stored at 5°C and extended the shelf life of pasteurized milk from 5 to 16 d without altering its properties. The versatility of RP-HPLC as a predictive and longevity tool has been demonstrated through these studies in various industries. Whereas the pharmaceutical example discussed finding degradation products for assessment, the food industry application uses RP-HPLC data to directly inform ways to extend shelf-life. The cases illustrated that RP-HPLC is necessary from a quality control and product development perspective.

8. Future Directions in RP-HPLC for Stability Testing (1 page)

Emerging Trends in RP-HPLC Technology

In-chromatography monitoring technology using reversed-phase high-performance liquid chromatography (RP-HPLC) has increasingly received attention in that it can better monitor incomplete reactions during the reaction process and improve sensitivity, while achieving on-line monitoring. In column technology, ultra-high-pressure liquid chromatography (UHPLC) is an important application, and its development mainly relies on using sub-2- μm stationary phase particles with higher column pressure to achieve faster separation and better resolution (Liu et al., 2010). This has allowed for small sample volume (e.g., 500 nL dialysate samples) analysis with retention times < 1 min for neurotransmitters like serotonin. In addition, it was found that capillary columns of 0.01 mm to 0.5 mm in diameter were effective in the separation of small amounts of peptides and proteins (Davis & Lee, 1992). There have been significant improvements in detector technology with sensitive low-dead-volume detection systems. One approach has been the detection of subnanomolar concentrations of serotonin from brain microdialysate samples (Liu et al., 2010), using techniques such as photoluminescence driven by electron transfer (PFET) and electrochemical detection. A novel method employs smartphones as compact and inexpensive fluorescence detectors in HPLC to measure multiple fluorescent compounds with distinct excitation wavenumbers simultaneously (Shamsaei et al., 2023). Data analysis tools have adapted to the needs of high-throughput studies. For automated workflows, such as retention time calibration, data extraction and quality criteria calculation for data curation, the modular toolkit HappyTools has been developed (Jansen et al., 2018). This software showed better precision and throughput than available commercial products.

But, of course, there is contradiction in the approaches to miniaturization. Some researchers have been working to develop more sophisticated



UHPLC systems, while others are investigating relatively simple, portable solutions. An example is a pump which utilizes no power to operate based on the controlled expansion of a pre-pressurized gas incorporated into a miniaturized, 6.7 kg liquid chromatography system (Chatzimichail et al., 2019). With growing column technology, detection technique, and data analysis software, RP-HPLC technology is still evolving in a rapid fashion, concluding the need in a short time line. These advancements propel the pursuit of nimbler, more sensitive, and more widespread chromatographic methods, enabling exciting possibilities across neuroscience, biopharma, and environmental fields.

Integration with Other Analytical Techniques

However, RP-HPLC has been integrated with mass spectrometry and proved to enhance the stability testing along with providing comprehensive pharmacokinetic information of pharmaceutical agents. Liquid chromatography–mass spectrometry (LC–MS) is a relevant technique in combination with RP-HPLC, providing an efficient platform for identification and characterization of drug substances and their degradation products. A similar approach was used for linagliptin (Ertaş, 2017) where a degradation-indicating RP-HPLC assay was developed and then used for LC-MS and UPLC-TOF/MS analyses in order to identify and characterize degradation products formed in stability studies. By integration of data that could identify the degradation compounds, the m/z values and fragmentation profiles of unknown degradation components were determined, and an overall degradation pathway was proposed for the drug. Importantly, RP-HPLC in combination with mass spectrometry has also found applications in areas other than pharmaceutical analysis. For example, a novel two-dimensional two-column LC/MS technique that combined chromatofocusing and NPS-RP-HPLC with ESI-TOFMS was developed to separate and analyze proteins derived from human breast epithelial whole cell lysates (Chong O et al., 2001). This application produces a 2-D map of proteins that

resembles an image of a 2-D gel, seeming to reflect the versatility in RP-HPLC-MS integration. Overall, RP-HPLC in combination with mass spectrometric and other detection methods has extended the power of stability studies and compound characterisation and the future looks bright in this area. Cromatografija molekūlāro grupu un masas spektrometrija (MS) ir katalizatora virzienā analīzes platformas kombinācija, kas ļauj ļoti augsta caurlaidspēja analīzēm, un piedāvā plašākas iespējas apgrūtinājuma produktu analīzei, nezināmu savienojumu identificēšanā un iespējam automatizētos konteinēšanas un mazo tilpumu aplikāciju izmantošanā, piemēram, farmaceitiskajā un citās augsto tehnoloģiju prognozējošā analīze.

Automation and High-Throughput Screening

About the Journal Submission Open All images Are Yoga Pants Safe During Helios Iliopsoas Asana? + Troubleshoot Wig Snags! + Inflatable Skin + More THURSDAY, MAY 20, 2021 These automation and high-throughput screening (HTS) techniques are revolutionizing stability testing in the pharmaceutical industry by enabling rapid, efficient, high-throughput assessment of drug candidates and formulations. These technologies greatly accelerated the drug discovery and drug development process, allowing researchers to rapidly screen hundreds of thousands of compounds, and other formulations, within a few days. The technology of microfluidic devices enables rapid, low-volume screening and directed evolution of biomolecules in a cell-free system (Contreras-Llano & Tan, 2018). Peptides and proteins can thereby be expressed in situ and on demand, supporting characterization of many formulations with regard to their stability. In terms of automation, informatics and multimodal analytical capabilities, the advent of automated, high-throughput fractionation procedures has also revolutionised natural product screening and structure-based multicomponent target analysis (Tu et al., 2010). Notably, HTS is now moving behind mere upthrouying. The aim of screening assays is generating a large amount of biological



data and this has prompted a growing effort in enhancing not only the screening capacity, but also the quality and the relevance of the data generated (Mayr & Fuerst, 2008). This evolution of biological test systems with the content and the quality is important for stability testing as they enable better predictions of drug stability.

Abstract: In summary, the combination of automation and HTS is changing the landscape for stability testing in pharmaceuticals by allowing for rapid screening and evaluation of large compound libraries, enhancement of biological data quality, and integration with other analytical techniques. Automated systems and quantitative HTS have generated millions of concentration-response curves from hundreds of thousands of assays in relatively short time frames (Michael et al., 2008), dramatically shortening the timelines of drug discovery and development. As these technologies advance, they will play a continually more vital role in assuring the stability and potency of pharmaceutical products.

9. Challenges and Limitations of RP-HPLC in Stability Testing (1 page)

Common Limitations in RP-HPLC for Stability Studies

Background: Although stable compounds are important to pharmaceutical analysis, there is no definitive guideline or regulation for these studies, Reverse-phase high-performance liquid chromatography (RP-HPLC) being one of the most applied in this field; however, RP-HPLC method development has limitations. This widespread analysis has always faced challenges such as extraction sensitivity, particularly for triterpenoids that do not have common UV absorption groups and chromophores and thus exhibit relatively low sensitivity in HPLC (Huang et al., 2023). This is achieved using improvement detection through chemical derivatization methods. Likewise, for the sugar analysis in mushrooms, because the refractive index detectors have low sensitivity and coeluting components easily interfere with it (Sławińska et al., 2020).

This is a significant issue if working with complex biological or pharmaceutical samples, which also leads to matrix interference. This leads to an inherent compromise, before optimizing MS data of a global extraction, pairing similar compounds can be in favour of protecting their attempt of fragmentation and also increasing their quantity (i.e., protonated molecule); on the opposite it may be a drawback due to the poor separation of structural similarities (for instance, a di-O methylated model with O methylated ones) (Huang et al., 2023). Therefore, overcoming this challenge, corona charged aerosol detection (CAD) was adapted to further increase the sensitivity and reproducibility (Sławińska et al., 2020). This requires broad optimization of chromatographic parameters, which are a classic characteristic of method development problems. Separation of ciprofloxacin hydrochloride and rutin was also ascertained by column, mobile phase composition and detection wavelength (Shamim et al., 2023). Similarly, DOE was applied to design the analytical method of linagliptin and its impurities to study the critical parameters of the Methode, which provide its robustness (Jadhav et al., 2017). RP-HPLC was concluded to be a potential method for stability studies if the problems related to sensitivity, matrix interferences and method development can be solved. All these limitations require new approaches to alleviate—from chemical derivatization techniques, rapid detection techniques as well as well-known analytical methods development protocols to render the RP-HPLC methods more amenable for pharmaceutical characterization.

Practical Challenges in Routine Use

Stability studies are carried out widely through reverse phase high-performance liquid chromatographic (RP-HPLC) methods in pharmaceutical analysis; however, they have practical complications.

RP-HPLC stability studies can be expensive due to the need for specialized equipment, shielding material, solvent, and columns. For instance,



(Jadhav et al., 2017) reported on the use of a Zorbax SB-Aq, column and gradient elution. This approach is based on specific HPLC devices and consumables. Moreover, this method generally consumes a large amount of organic solvents, causing high operating costs and environmental issues (Yabré et al., 2018). Time is an important parameter in the RP-HPLC stability studies. The other study (Jadhav et al., 2017) highlighted long method development through design of experiment that was applied to optimize a few key method parameters. Routine analysis is also time-consuming, taking minutes to run (Fouda et al. 1991; Shamim et al. 2023). Meanwhile, RP-HPLC methods remain developed and implemented with painstaking expertise to carry out the stability work. Introduction Developing methods is a rather complicated task per ICH guidelines, where reviewing the method parameters, method validation and interpretation of the results requires a comprehensive understanding of the characteristics of a method and its complexity which can make it a challenge for the scientists (Jadhav et al., 2017). For example, though the identification of degradation products usually requires additional knowledge of MS-based methods such as LC-MS along with UPLC–TOF/MS (Jadhav et al., 2017). Consequently, while RP-HPLC is probably the most powerful method for stability study, routine application based on this technology is not only complex, but also too costly and time-consuming as a trained technician has to carry out the study. In pharmaceutical product development and quality control, stability studies must be thoughtfully designed and painstakingly executed, taking into account the numerous factors that are related to product stability.

Solutions and Recommendations

Automation and advanced technologies provide the potential solutions to bridge these constraints across a wide range of applications, such as regenerative medicine, construction, scientific research, and manufacturing. Automation at software and hardware levels can solve reproducibility and up-scaling challenges in

regenerative medicine. The robustness and potency of therapeutic cellular other products was explored by the use of artificial intelligence models, machine learning techniques, automated liquid handling, and automated cell expansion bioreactor systems (Doulgkeroglou et al., 2020). Likewise, in the field of construction, systems that support decision-making like AUTOCOP apply analytical hierarchy methods that analyze whether automated systems are preferable to traditional processes based on a variety of considerations, including economic, technological, and safety criteria. (Hastak, 1998). Automation is there to make things smoother and faster for people, but sometimes an unmanaged approach can reduce the quality of life of them — if the automation is something you use on a basis. Robotic process automation (RPA) in purchasing processes must be carefully implemented for uplifting effects on consumer satisfaction and engagement (Gavrila Gavrila et al., 2023). However, it underscores the need to look beyond proactive technological approaches with consideration for end-user satisfaction. To summarize, these fields can be greatly enhanced through the automation and advanced processing technology. But to be successful, implementation must be well planned, carefully consider many factors, and ensure the needs of the end user are focused on. These include early adoption of automation throughout the development spans, implementation of decision support systems, and data-rich experimentation bolstered by integrated process analytical technology which enhance processes to be more robust, efficient and effective (Doulgkeroglou et al., 2020; Hastak, 1998; Nambiar et al., 2022).

SUMMARY

- RP-HPLC is an effective and widely used analytical technique in the pharmaceutical field for the separation and quantification of different constituents like drugs, impurities, and degradation products. The stability studies serve a critical role in ensuring drug product quality and determining the shelf life of a drug by assessing how environmental factors —



temperature, humidity, and storage conditions — would impact the drug potency, safety, and efficacy over time. Stability-indicating RP-HPLC methods are particularly useful in such studies, as they can accurately measure drug concentration changes over time and estimate the content of degradation-related impurities. The objective of method development is to optimize the chromatographic conditions such as optimizing mobile phase composition chosen for analysis, column selection, and a choice of a detector to achieve maximization of analytes separation and detection. ICH guidelines direct the validation of these methods, which establishes their reliability, accuracy, as well as suitability to the purpose. Long-term stability studies administered under reverse phase high performance liquid chromatography demonstrate how these methods can be used to excel drug shelf life prediction through degradation rate extraction. RP-HPLC is one of the most common and widely used methods; however, it also suffers from poor sensitivity, matrix interference and method development. Recent advancements in RP-HPLC technology are centered on creating better column performance, improved detection, and comprehensive software for data analysis. The advent of integration with other analytical techniques, especially mass spectrometry, has greatly expanded the possibilities with stability testing and compound characterization. These advancements provide new capabilities that were previously unattainable due to data and resource limitations—which ultimately streamlines the drug development process substantially with the increase in automation, high-throughput screening, and multiplex approaches in stability testing.

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