INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES
[ISSN: 0975-4725; CODEN(USA): IJPS00]
Journal Homepage: https://www.ijpsjournal.com

Review Paper

Revolutionizing Stability-Indicating Analysis: Advanced RP-HPLC Strategies for Pharmaceutical Excellence

Harshad Tanpure*, Dr. Vishwas Bahgat, Dr. Deepak Kardile, Dr. Rajkumar Shete, M. M. Karne

Rajgad Dnyanpeeth's College of Pharmacy, Bhor, Pune - 412206

ARTICLE INFO

Published: 27 May. 2025
Keywords:
Revolutionizing Stability, Indicating Analysis, RP-HPLC, Pharmaceutical Excellence
DOI:
10.5281/zenodo.15512978

ABSTRACT

Stability-indicating analysis is vital to pharmaceutical quality control for establishing the safety, efficacy, and quality of pharmaceutical drug products until their expiration date. Reversed-phase high-performance liquid chromatography (RP-HPLC) has recently become an important tool in stability testing because of its high selectivity, sensitivity, and robustness. Abstract/Short: This review focused on recent developments in RP-HPLC approaches for stability-indicating analysis to emphasize their importance in pharmaceutical excellency. Abstract The principles of stability-indicating analysis along with regulatory guidelines, classification of degradation pathways of drugs, and importance of these studies in drug development are discussed. This review outlines the main advantages of RP-HPLC in stability testing, which include selective separation, high sensitivity and excellent ruggedness. In addition, we covered the challenges of stability testing and provided a solution and response with RP-HPLC through optimized separation and sensitive detection and validation of the methods described. The development of recent RP-HPLC columns, including sub-2 µm packing materials for higher-resolution applications and stationary phases with specific interaction sites for improved selectivity, has greatly expanded the separation space and analytical flexibility. The results indicate that advances in detection methods, specifically mass spectrometry, along with new strategies like smartphone-based fluorescence detection, provide further specificity and sensitivity for stability-indicating analysis. Method validation according to the ICH and USP guidelines is important, as illustrated by case studies: development and validation of stability-indicating RP-HPLC methods for the estimation of betamethasone acetate and betamethasone dipropionate simultaneously; design and validation of a stability-indicating RP-HPLC method for estimated estimation of calycosin-7-glucoside in Corydalis species;

***Corresponding Author:** Harshad Tanpure
Address: *Rajgad Dnyanpeeth's College of Pharmacy, Bhor, Pune - 412206*
Email ✉: harshadtanpure2001@gmail.com
Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

analysis of coexisting standard products and stability studies; and HPLC separation of flufenamic acid and its impurities by a rapid method validated in an effective selection of experimental conditions. Lastly are the applications of RP–HPLC in stability studies in terms of pharmaceutical product, degradation product and regulatory compliance approaches. This review wraps up by recapping the major developments in RP-HPLC for stability-indicating analysis and what it means for continuous improvements in pharmaceutical quality and some trends and future prospects PHPLC in these regards.

INTRODUCTION

Overview of Stability-Indicating Analysis

Stability Indicating Analysis is an important part of pharmaceutical quality control for the detection and quantification of drug substances and their degradation products. This method of analytical assessment maintains the integrity and stability of the pharmaceutical substances for the entire shelf life of the product (Fawzy and Kamel, 2023; Nekkalapudi et al., 2023). Development of stability-indicating methods are carried out by spectrophotometric and high-performance liquid chromatography (HPLC) methods. For example, (Gamal Fawzy & Kamel, 2023) reported thesseed capablehundredth spectrophotometric methods for the on-volley reassessed of onMomolnupir specify Duringsthydroxylated monomerinepurdence. They are characterized by linearity, sensitivity, and environmental sustainability. Likewise, (Nekkalapudi et al., 2023) described a rapid and simple isocratic HPLC method for ixabepilone assay in active pharmaceutical ingredients and in injection-dosage forms. However, it is worth noting that the analysis used in pharmaceutical research has progressed beyond the historical line of reasoning. The trend of using mass spectrometry, and especially of ultra-high-resolution Fourier transform instruments, is rising (Deschamps et al., 2023). With high resolving power, mass accuracy, and dynamic range, these methods provide reliable molecular formula assignments and trace analysis in complex mixtures. To conclude, stability-indicating analyses are integral to the control of pharmaceutical quality and safety. Green, efficient, and advanced analytical methods are further evolution of drug analysis methods which supply the accurate and precise results for quality assured potent pharmaceutical products and patient safety.

RP-HPLC in Stability Testing

Method Development: RP-HPLC is selected over other approaches such as isocratic, normal phase HPLC etc. due to its higher selectivity, sensitivity & robustness making it the preferred choice for stability-indicating analysis. These benefits have been shown in several works for the analysis of different pharmaceutical compounds and natural products. RP-HPLC is a selective technique, which validates the genuineness of the individual drug from their impurities after varying stress conditions. A representative example is a stability-indicating HPLC method, which indicated separation of canagliflozin from the degradation products, and the peak purity values suggesting that the drug was homogeneous in all the test degradation conditions (Azhakesan & Kuppusamy, 2023). Likewise, curcuminoids can be separated and detected using short HPLC method, which is capable of detecting these compounds in natural extracts, commercial dosage forms and forced degradants in a single mixture (Mohammed et al., 2023) RP-HPLC has a high sensitivity with low limits of detection and quantification. The method synthesized for pterostilbene, for example, realized an LOD of 2.65 ng/g and an LOQ of 7.95 ng/g (Haq et al., 2023). The sensitivity was also higher for L-ascorbic acid in the HPLC-ECD method with LOD of 0.0043 μg/mL (Wu et al., 2023). Another important characteristic of RP–HPLC is the

robustness. The overall %RSD obtained was around 0.50%, confirming the robustness of the Canagliflozin method (Azhakesan & Kuppusamy, 2023). This curcuminoid method applies Monte Carlo simulation to assess operability and to enable simultaneous detection from different samples (Mohammed et al., 2023). Moreover, cefoperazone method avowed its efficiency with the accomplishment of system suitability regarding robustness and ruggedness (Al-Hakkani et al., 2023). Conclusion RP-HPLC combines several desired properties such as selective separation, high sensitivity and ruggedness thereby making it suitable for its favourable use in stability indicating analysis of pharmaceutical and natural products. This allows for assured quantification of the compound and its degradation products leading to reliability of stability studies [2].

Purpose and Scope of the Review

RP-HPLC techniques: There have been a number of improvements in recent times owing to which RP-HPLC techniques are widely employed for stability analysis for efficacy of pharmaceuticals. Development of stable and rapid methods has improved drug product quality control and characterization. Among them, the application of imaged capillary isoelectric focusing (icIEF) for monitoring adeno-associated virus (AAV) gene therapy vectors represents a significant advance. Such a method represents a greater depth of knowledge on capsid viral protein charge heterogeneity from previous approaches relying upon HPLC and capillary electrophoresis (He et al. 2023). The icIEF method has also been demonstrated to be stability indicating and establishes a correlation between increase of acidic species with associated transduction efficiency loss delayed by deamidation. However, the adoption of quality by design (QbD) approaches for RP-HPLC method development has made the process more systematic and the results statistically valid. For example, simultaneous estimation of ciprofloxacin hydrochloride and rutin was successfully evaluated taking less number of design points and experimental runs by using Box-Behnken design (Shamim et al., 2023). Such an approach not only improves the result of the analysis but also gives a deeper insight into the factor-response relationship. In summary, recent trends in the stability analysis of drugs using RP-HPLC methods have provided better overall characterization and have correlated to biological activity at an advantageous efficiency level improved significant. New tools like icIEF and QbD have been integrated into the growing analytical toolkit for the development and manufacturing of characterized pharmaceutical products.

Page 2: Basics of Stability-Indicating Analysis

Definition and Regulatory Importance

Stability-indicating analysis is an important part of pharmaceutical quality control, as it is conducted to quantitatively estimate active ingredients and degradation products in a drug formulation. Stability-indicating methods must be able to separate the active from degradation products when both the drug substance and products are subjected to environmental conditions over time (Kanagaddi et al., 2024; Ramireddy & Behara, 2023). They are important approaches used for product life cycle and providing assessment of drug quality, safety, and efficacy [6]. Various guidelines by International Conference on Harmonization (ICH), United States pharmacopeia (USP) and Food and Drug Administration (FDA) exist for developing and validation for stability Indicating methods. The stability of the prepared batches was determined based on the requirements from ICH guidelines

Q1A(R2) [1] and the validation of the analytical methods from the Q2(R1) [2] guidelines (Ramadan et al., 2023; Ramireddy & Behara, 2023). FDA guidance documents also highlight the need for stability-indicating methods for both approved drugs (Fernandez Lynch et al., 2023) and drugs approved through accelerated pathways. Notably, while chemical analysis is the main aim of typical stability-indicating methods, major efforts would strive for expansions of the current approaches. Example: current FDA initiatives such as artificial intelligence and natural language processing for regulatory review218 may influence future efforts in analyzing and interpreting stable data.28 (Gray et al., 2023) Furthermore, for some drugs like peptides and proteins, conventional stability-indicating methods are not sufficient leading to the development of alternative strategies to monitor their long-term stability and safety (Wu et al. In summary, a stability-indicating analysis is an essential step for both development and quality control of a pharmaceutical, and here itis governed primarily by both the ICH and USP regulatory frameworks, and the FDA. Such methods need to be in accordance with strict validation requirements to confirm their accuracy, precision, and specificity for the quantification of drug substances and products over time. The methods of analysis applicable for stability-indicating purpose must possess certain features so that any change in the stability of the drug under investigation can be detected unambiguously. These methods need to have specificity, selectivity, and robustness. Stability was indicated by the development of an HPLC method, it must be specific, as done in the case of Canagliflozin. The peak purity values of canagliflozin obtained through this method were indicative of homogeneity under various degradation conditions (Azhakesan & Kuppusamy, 2023) Separating canagliflozin along with its degradation products were successfully achieved through this method under different exceed limits of degradation conditions. Likewise, HPLC also exhibited a high degree of specificity for ibuprofen and phenylephrine as it resolved the drugs and their degradation products (Kelani etal.,2023). Specificity is directly linked to selectivity, making this quality necessary to differentiate between analytes and other potentially interfering materials. Apart from separately excising the drug and degradation products of both phenylephrine and ibuprofen, respectively, the studies (Kelani et al., 2023) are further validated; the studies confirmed that the separation of the drugs and their degradation products are satisfactory which elucidate the selectivity of the method. In both studies (Azhakesan and Kuppusamy, 2023; Kelani et al., 2023), the reliability of the method under variable circumstances is also analyzed, termed robustness. Finally, stability-indicating analysis methods should be selective (or specific enough) to be able to separate the drug from its degradation products, selective to be able to separate the analyte from potential interferent under the condition of the intended use, and robust to provide consistent results under varied conditions [20]. All these attributes are important for an accurate evaluation of the stability of the drug and they are generally carried out using ICH guidelines, which is the case of all the studies discussed.

Types of Drug Degradation

Drug degradation can occur by different ways like chemical, physical and microbiological. For the purposes of this discussion, the most important process is chemical degradation, meaning the breakdown of a chemical bond, like oxidation, hydrolysis, or photodegradation. For many drugs and excipients, oxidative degradation occurs and is thus a concern. Polysorbates are one example of

a surfactant in a biologic that can be prone to oxidative degradation in the presence of light, residual metals, peroxides, or heat (Weber et al., 2023). It leads to a loss of efficacy or formation of a particle in a pharmaceutical formulation. Hydrolysis is a significant route for chemical degradation. For polysorbates, hydrolysis may be chemical or enzymatic, the latter often coming from host cell proteins under pharmaceutical conditions (Weber et al., 2023). Photodegradation, that is the light-mediated degradation process, is another important drug stability consideration. As an example, chloroquine phosphate (CQ) has been shown to undergo direct photodegradation under simulated sunlight, and the quantum yield of degradation is also pH-dependent (Xiang et al., 2023). Notably, some degradation processes were linked to each other. In newer research, for instance; photodegradation of ascorbic acid in juices leads to a series of reactions which extends the degradation of ascorbic acid during storage (Basak et al., 2023). Likewise, sulfamethoxazole degradation has studied a combination of the two mechanisms involving chlorine oxidation and UV photodegradation demonstrating the interactions between different mechanisms (Queral-Beltran et al., 2023). Overall, while the many pathways through which a compound can degrade are known, understanding them is vital for providing stable and effective drug. Although this overview largely concerns itself with chemical degradation mechanisms, physical (e.g., polymorphism) and microbiological degradation is also critical to drug stability but neglected by the literature.

Role of Stability Studies in Drug Development

Stability studies are one critical element that needs to be studied in terms of Drug development from pre- formulation to post- marketing monitoring for safety and efficacy of pharmaceutical products. Drug-excipient compatibility studies (DECS) are the key studies in pre-formulation to choose the right excipients to achieve an optimum stable formulation. To overcome these issues, we proposed a novel vial-in-vial approach compatible with current study strategies and high throughput screening of excipients. The results obtained were quite discriminatory with this method resulting in significant degradation compared to standard methods making it possible to decrease the drug development time by eliminating late shocks in the stability studies (Jain & Shah, 2023). Safety surveillance is a key aspect of protecting the health of trial participants and providing reassurance that identified risks remain minimal after the product receives marketing authorization. This includes the methodical gathering, evaluation, and way of adverse outcomes by investigators and sponsors as well periodic summary safety reports that develop with the benefit threat of the investigational product to be sent to health authorities (Samara et al., 2023). While stability studies are essential, it is worth nothing that the majority of drug attrition in clinical trials (about 90%) is due to unexpected toxicity issues discovered preclinically. This emphasizes the need for knowledge on drug–biological target interactions and appropriate computational methods for assessing protein–ligand interactions and predicting toxicity (Amorim et al., 2024). Stability studies are fundamental to drug development from pre-formulation to post-market monitoring [32] They are useful in establishing product safety and efficacy, guiding formulation decisions and to communicating potential hazards to regulators. It is suggested that advancements in methodologies — including the novel vial-in-vial approach in this study as well as computational methods that could potentially predict toxicity before drug testing — can ultimately benefit the drug development process and increase success rates.

Page 3: Fundamentals of RP-HPLC

Reverse-Phase Chromatography

RP-HPLC is a commonly applied separation technique utilized in the separation of analytes based on the hydrophobic interaction between a stationary phase and the analytes. The stationary phase mostly consists of hydrophobic alkyl chains (e.g., C18) immobilized to silica particles, while the mobile phase is mostly composed of water and organic solvent (Kinoshita et al., 2023; Mallik et al., 2023). RP-HPLC operates by a separation mechanism where the sample components partition differently into the hydrophobic stationary phase versus the more polar mobile phase. Non-polar compounds interact more strongly with the stationary phase and are retained longer, polar compounds elute earlier. After this, it enables the elution of complex mixtures on the base of the relative hydrophobicity of the compounds (Ferencz et al., 2023; Mallik et al., 2023). Notably, newer phases, such as Sil-Ala-C12 derived from amino acids, can behave as both RS and HILIC phases, making possible the use of a dual-function column. Such versatility may shorten the expense of purchasing columns and facilitate the scalability of methods (Mallik et al., 2023). Moreover, chiral stationary phases (CSPs) provide an orthogonal selectivity using RP-HPLC mode for RP-HPLC separation of structurally similar compounds (e.g., enantiomers) (Ferencz et al., 2023; Zhong et al., 2023). In summary, RP-HPLC separation using hydrophobic interactions provides flexibility for a wide variety of compounds. This multilabel single-cell strategy is a powerful and versatile tool that can be adapted to a wide range of applications, such as pharmaceutical analysis, environmental monitoring, and metabolomics studies (Pérez-López et al., 2023; Voronov et al., 2023; Zhong et al., 2023).

Key HPLC Components

HPLC is a shared system made up of multiple items that work together to isolate and analyze chemicals.

Pump: It supplies the mobile phase to the system at a specific flow rate. Such as, (Najmi et al., 2023) used 1 mL/min as flow rate for the caffeine analysis Separation occurs in the column, which is usually filled with particles of stationary phase material. Different column types were employed including C18 (Haas et al., 2023; Najmi et al., 2023), C8 (Ramireddy & Behara, 2023), and porous graphitic carbon columns (Sun et al., 2023). An injector injected the sample into the mobile phase immediately before the column. (Shamsaei et al., 2023). Compounds eluting from the column were detected by the detector. The common detectors are UV-vis (Azhakesan & Kuppusamy, 2023; Najmi et al., 2023; Shamim et al., 2023), fluorescence (Shamsaei et al., 2023), and photodiode arrays (Azhakesan & Kuppusamy, 2023; Haas et al., 2023). The data system picked-up detector signals and generated chromatograms for further analysis. MOCCA (Haas et al., 2023) is a Python package designed for HPLC data analysis. Notably, we have recently developed a new HPLC system called "phase separation mode" utilizing multiphase flow as the eluent (Kinoshita et al., 2023). An Inexpensive Smartphone-Based Fluorescence Detector (Shamsaei et al., 2023) developed a new smartphone-based fluorescence detector as a low-cost alternative to conventional detectors. To summarise, although the fundamental parts are similar in every HPLC system, continuous innovation is happening to improve the separation, detection, and data presentation aspects of chromatographic systems. These components work together to enable accurate and reproducible chemical analyses for a variety of applications.

Critical Parameters in Method Development

As shown in the attached papers, the most critical parameters in HPLC method development are as shown below-- Method development for HPLC consist of several important factors such as mobile phase composition, column selection, flow rate, temperature and detection wavelength etc. Research has shown that these factors matter. It is very important that the mobile phase be made up of the compositions that lead to the best separation. As mobile phase (Mohammed et al., 2023) used acetonitrile-phosphate buffer (54:46 v/v), while as (Rathee et al., 2023) used methanol/water (80:20% v/v). Column selection is also an important aspect with some studies using Shim-pack Solar C8 (Rathee et al., 2023), Kromasil C18 (Elsheikh et al., 2023), and X-Bridge C18 (Menda et al., 2024) columns. The flow rate (1 ml/min) and column temperature (30–40 °C) were also identified as critical method parameters (Mohammed et al., 2023; Rathee et al., 2023). Interestingly, a few studies have also used experimental design approaches to find the best combination of these parameters. For example, (Rathee et al., 2023) utilized the Bo–Behnken design to optimize the mobile phase composition, Junaid et al (Hussain et al., 2023) applied Taguchi model and central composite design for optimization of mobile phases ratios and column temperatures. This shows the increased interest in the use of statistical approaches for method optimization (Hussain et al., 2023; Rathee et al., 2023). Abstract Mobile phase composition, column selection, flow rate, temperature, and detection wavelengthare some of the most important parameters that need to be carefully considered while developing anHPLC method.

Page 4: RP-HPLC in Stability-Indicating Analysis: Key Benefits

Sensitivity and Specificity

RP-HPLC is advantageous for stability-indicating assay due to its ability to trace degradation products detection from complicated matrices. Because of its high sensitivity and specificity, it is a perfect technique for this end. Sensitivity for low levels of degradation products was good with RP-HPLC detection. A recent study regarding canagliflozin reported linear range of 12.6-37.9µg/mL and high precision (%RSD<0.66%) (Azhakesan & Kuppusamy, 2023). Likewise, an RP-HPLC method developed for an analysis of pterostilbene was reported with a high degree of sensitivity (LOD 2.65 ng/g, LOQ 7.95 ng/g) (Haq et al., 2023). These low detection limits allow for the detection of trace amounts of degradation products. In contrast, RP-HPLC is specific in not only separating components in its original matrices but also separating when a sample has been in a complex matrix. For instance, stability-indicating HPLC method was developed which separated canagliflozin from degradation products under different forced degradation conditions (Azhakesan & Kuppusamy, 2023) Such specificity is important because we need to accurately quantify these at-pharmaceutical ingredients, and any degradation products, as this allows us to perform stability studies. To summarize, the high specificity and sensitivity of RP-HPLC makes it an ideal candidate in stability-indicating analysis. The LSO-RP technology can separate degradation products and other matrix components from the active ingredient while detecting only trace amounts of these degradation products, which makes this technology suitable for obtaining unbiased results from challenging pharmaceutical formulations or complex biological samples.

Challenges in Stability Testing

Stability testing for pharmaceuticals presents unique challenges, such as interference from degradation products, matrix effects and the need

for low-level impurity detection. It has been demonstrated (Williams et al., 2023) that matrix effects can be one of the most significant contributors affecting the accuracy, sensitivity, and reliability of the separation techniques used for stability testing. These effects can cause either ion suppression/enhancement or interference with the analyte signal during different steps of the analytical workflow that represent a major challenge to the analytical process. Proposed solutions include a change in an ionization type used, improved extraction and clean-up methods, optimization of chromatography conditions, and the use of corrective calibration methods (Williams et al., 2023). Notably, the degradation products formed during forced degradation studies may coincide with drug metabolism [pathways producing similar entities (Gumieniczek & Berecka-Rycerz, 2023). While this overlap creates challenges, it also presents opportunities where metabolic data can provide safe levels of degradation impurities, leading to enhanced pharmaceutical product quality. Nevertheless, some classes of drug (peptidic compounds like glutides and polar drugs such as gliflozins) are prone to give rise to specific chromatographic problems which must be overcome (Gumieniczek & Berecka-Rycerz, 2023). In order to manage these challenges, various advanced analytical techniques and data analysis approaches have been developed. MassChemSite, for example, has been referred to as an attractive informatics platform for the analysis of forced degradation studies utilizing LC-MS/MS and UV data (Bonciarelli et al., 2023) and for the automated identification of structures of degradation products. Moreover, LC-MS has played a central role in the identification, quantification, and clearance tracking of individual host cell proteins, which constitute process-related impurities in biotherapeutics (Guo et al., 2023). Development of analytical methodologies and data analysis tools are of utmost necessity to meet the challenges embedded in stability testing and ensuring the quality and safety of the pharmaceutical dosage forms.

How RP-HPLC Addresses These Challenges

There are numerous benefits that RT-HPLC provides in overcoming the obstacles associated with the robust separation of degradation products, with better detection and also validation of the method. By careful optimization of the chromatographic conditions, robust separation of the degradation products is achieved using reversed-phase HPLC (RP-HPLC). It enables gradient elutions as well as the use of optimum composition and rate of mobile phase for the effective separation of closely similar compounds and degradation products (Mohammed et al., 2023; Ramireddy & Behara, 2023). The column selection, temperature, and pH can also be adjusted to increase the separation power (Azhakesan & Kuppusamy, 2023; Shamim et al., 2023). More sensitive detectors (if using UV, fluorescence, and mass spectrometry are used) enables better detection. Sensitive and specific detection of analyte can be achieved using UV detection at optimized wavelengths (Ramireddy & Behara, 2023; Shamim et al., 2023). Fluorescent compounds provide an even more sensitive detection method utilizing fluorescence (Almutairi et al., 2023). Then the separated species can be identified based on mass spectrometry (He et al., 2023). Method validation was carried out according to the guidelines prescribed by ICH for a rigorous validation of the technique so that reliable and reproducible results can be obtained. Analytical methods used to evaluate the rooted plants are consequently most often characterized regarding key validation parameters, such as specificity, linearity, accuracy, precision, robustness and stability indicating function (Azhakesan & Kuppusamy, 2023; Ramireddy &

Behara, 2023; Shamim et al., 2023). Quality by Design (QbD) strategies are increasingly implemented for the systematic optimization of critical method parameters to develop robust method (Azhakesan & Kuppusamy, 2023; Hussain et al., 2023; Mohammed et al., 2023). Notably, RP-HPLC has also been used in some tests for difficult separations of chiral compounds (Al-Sulaimi et al., 2023) and complex mixtures of natural products (Mohammed et al., 2023). This method has also been adapted to conduct high through-put assays in 96 high-throughput plate format (Almutairi et al., 2023). Although RP-HPLC answers important analytical challenges via separation with optimal optimization and detection with high sensitivity and validation with a high level of scrutiny. Recent developments in column technology, instrumentation, and approach to methods development continue to improve their utility in pharmaceutical analysis and quality assessment.

Page 5: Innovations in RP-HPLC Columns and Stationary Phases

Modern Column Technologies

However, substantial progress in the separation capabilities and analytical performance of RP-HPLC columns and stationary phases has inspired this review through recent innovations. Recently, these functionalized groups have been introduced in the manufacturing of stationary phases for HPLC, enhancing the versatile character of HPLC columns. In one example, a new stationary phase (Sil−Ala−C12), which was prepared by immobilizing dodecylamine and alanine on silica, exhibited good reversible-phase HPLC and HILIC separation (Mallik et al., 2023). This phase obtained baseline separation of difficultβ-andγ-isomers of tocopherol and performed multi-analyte analysis, which conventionally required separate columns, thereby limiting column purchase costs. Meals and certain others, that demonstrate varied stationary phase separation capability for distinct classes of analytes, across comparative studies. For instance, extracts of Pinus cembra heartwood revealed antifungal dihydropinosylvin monomethyl ether and pinosylvin monomethyl ether which were separable on pentafluorophenyl (PFP) columns, but not on C18 materials (Alperth et al., 2023). This underscores the need to choose the right stationary phases to solve some specific analytical issues.

Abstract: New column technologies have allow RP-HPLC to do much more than it used to. Newly designed stationary phases with designed interaction sites and the utilization of several different column chemistries (C18, phenyl, PFP) have improved the separation efficiency and analytical flexibility. This allows for more rapid and affordable analyses across a range of applications from complicated natural product extracts to pharmaceutical small molecules.

Particle Size and Resolution

The invention of sub-2 μm particles for RP-HPLC columns has greatly increased the chromatographic separation resolution and speed. This reduction in particle size results in much faster analysis times and increased peak resolution (compared to the 5 μm particles traditionally used). The ISRS ratio decreases with the reduction of the RP HPLC bead size 3 and to date many others have shown the advantages of small particle sizes for RP-HPLC. As an example, (Haq et al., 2023) explained an application with a column with 5 μm particles that provided fast analysis with a 2.54 minutes pterostilbene retention (Haq et al., 2023). On the other hand, (Ramireddy & Behara, 2023) took advantage of a smaller particle size (5 μm) in a column, resulting in a 15-minute run time for simultaneous analyses of the two compounds

(Ramireddy & Behara, 2023). This implies more complex separation in thing times when reducing the particle size. What's remarkable is that in general, a smaller particle leads to better performance but some studies have also been used with larger particle sizes. They reported wide column noise, although good separation of ciprofloxacin and rutin was obtained using a 5 µm particle column (Shamim et al., 2023). In some applications, this suggests that bigger particle sizes can be countered by optimal mobile phase compositional conditions. Thus, the ongoing miniaturization trend in RP-HPLC has yielded major advances towards resolution and speed by making it possible to utilize sub-2 µm particles. Discovering method development taking into consideration various other aspects apart from particle size, still plays a significant role in enhancement of chromatographic performance. The particle size should be selected according to the underscores separation requirements as well as instrumentation capabilities.

Stationary Phase Modifications

The past few years have witnessed the recent RP-HPLC column and stationary phase innovations which have resulted in the development of stationary phases for dedicated degradation pathway and formulation studies. Recently, a new CSP enabled by porous organic cages (POCs), has been reported to achieve effective separation of chiral compound and positional isomers (Gong et al., 2023). This POC-based column showed enantiomeric separation that was complementary to commercial columns and was able to separate racemic compounds that another column was not capable of separating. An amino acid-derived stationary phase (Sil-Ala-C12) possessing targeted interaction sites has been established with high-performance separation capabilities in both reversed-phase HPLC and hydrophilic interaction chromatography (HILIC) (Mallik et al., 2023). It can separate difficult isomers, and be used to reduce overall column purchasing cost because the column can have multiple uses. Oddly enough, some studies have identified counterintuitive behavior in the performance of these columns. As an example, hysteresis of retention times and enantioselectivity was detected in the reversed-phase mode as function of composition of methanol/water mixtures on amylose-type columns (Dobó et al., 2024). Such findings can pertain to enantioseparation as the three-dimensional arrangement of the amylose column can transform when exchanged from the polar organic to the reversed-phase mode1–3. The recent innovations in stationary phase design exemplify possibilities to realise custom-tailored chromatographic columns to help overcome challenges such as peak tailing, excessive band broadening, and low analyte resolving power to optimize overall chromatographic performance. Multipurpose columns are emerging along with some unique behaviors from columns such as those utilized for the separation of degraded chemical constituents or the separation of analytes reference to an internal standard utilized in decomposition studies or formulation analysis methods and offer new directions for HPLC methods optimization.

Page 6: Mobile Phases and Separation Optimization

Solvent Selection and Composition

In many analytical and bioanalytical applications, the choice and composition of mobile phases are essential to optimizing separation and extraction. The efficiency and selectivity of separation are highly dependent on organic solvents, buffers, and their pH. There are common places to have Organic solvents as the extraction and separation of various compounds. As an example, in the

extraction of anthocyanins from strawberry, methanol and methanol: water mixtures were found to yield the highest anthocyanin and antioxidant activity, while acetone resulted in extracts with poor stability (Taghavi et al., 2023). Different solvents affect not just the yield, but also the extracted compound profile, as shown here by the predominance of different anthocyanins in various solvents [51, 52]. While buffers are important to keep the pH stable and suitable for the separation. Yet the nature of the buffers can influence analytical processes in the most unanticipated manners. In nanozyme-based assays, for instance, imidazole contained in commercial substrates could deactivate the catalytic activity of the nanozymes. In contrast, minor changes in the substrate buffer composition comprising of simple additions of high concentrations of NaCl or NH4Cl to citrate, MES, HEPES or TRIS buffers led to a drastic improvement in the oxidizing properties of the Prussian Blue nanozymes for 3,3'-diaminobenzidine (Khramtsov et al., 2023). One parameter that is vital in the optimization of separation is the pH of the mobile phase. Either way, interactions of the buffers with the analytes should be taken into account. Mn (IV) may be reduced to Mn (III) in manganese oxide systems by common buffers used to control pH (Good's buffers; Hausladen & Peña, 2023). Likewise, in atmospheric chemistry weak acid and baselike CO2, NH3, and organic acids are important in the buffering of acidity, and these buffering effects depend on both the pH range and environmental conditions (Zheng et al., 2023). Finally, the choice and adjustment of mobile phase components, organic solvent, buffer, and pH, must be performed to achieve the best separation and extraction. To generate robust and efficient separation methods, researchers should consider (1) the potential interactions between mobile phase components and analytes and (2) the specific needs of their analytical techniques.

Gradient vs. Isocratic Elution

Gradient elution typically provides better separation and resolution than isocratic elution for complex stability studies, particularly for multiple analytes or degradation products.

Gradient elution advantages for stability studies.

This enables the gradual variation of the composition of the mobile phase with time, allowing sufficiently retentive elution of closely eluted compounds (Neumann et al., 2024; Niezen et al., 2023). This is especially useful for isolating degradation products from the parent compounds. It may also enhance peak shape and resolution, particularly for late-eluting compounds (Abusultan et al., 2023; Niezen et al., 2023). This allows for the separation of low-to-high polar compounds in a single run (Neumann et al., 2024; Ramireddy & Behara, 2023) This allows for shorter analysis times than what is possible using isocratic approaches, especially with complex samples (Jeelani & Kouznetsova, 2023). But isocratic elution still applies in some cases: e.g., less complex samples (fewer analytes, straight chained compounds with similar retention) (Nekkalapudi et al., 2023). Gradient methods may be comparatively more sensitive to instrumental differences when transfer of methods between different instruments is important (for example, if it is the case that they are swapped in production) (Niezen et al., 2023). Emphasis is made on easy and simple method development (Nekkalapudi et al., 2023) In summary, while gradient elution is commonly favoured for complex stability studies due to their superior ability to separate components, there are many factors to consider when deciding between gradient vs isocratic

elution, including: ability to resolve the desired components of interest, matrix complexity, and robustness of retention time to various changes in ionic strength. In stability-indicating methods, superior separation of degradation products and impurities from the main compound generally provided by gradient elution (Jeelani & Kouznetsova, 2023; Ramireddy & Behara, 2023).

Additives and Modifiers

Mobile phase additives and modifiers have significant importance in improving the separation efficiency and peak shapes in chromatography. There are also many acidic /basic compounds, salts and other additives that directly influence the performance of chromatographic separation. Commonly used volatile salts at neutral pH in aqueous mobile phases for native proteins and protein aggregates characterization in size-exclusion chromatography coupled with native mass spectrometry (SEC-nMS) are very troublesome. However, high salt concentrations can retroact on the gas-phase analysis of labile protein complexes. In an effort to overcome the limitation, researchers have studied narrow SEC columns (1.0 mm internal diameter) run at low flow rates (15 µL/min) leading to higher protein-ionization efficiency and the realization of lower abundant impurities and higher-order structures up to 230 kDa (Ventouri et al., 2023). In this regard, ionic liquids have been used as mobile phase modifiers in the reversed-phase liquid chromatography (RPLC) mode with very promising results. For example, 1-butyl-3-methylimidazolium tetrafluoroborate (BMIM[BF4]) used in acetonitrile: phosphate-buffer-based mobile phase led to the convenient separation of nicotine and cotinine which normally are difficult to analyze owing to the polar basic character of these compounds (Axente et al., 2023) In summary, appropriate mobile phase modifiers/additives may do wonders to improve peak shape in chromatographic separations. Scientists have shown that certain volatile salts, ionic liquids, and other additives can mitigate many of the difficulties associated with certain analytes, leading to improved chromatographic performance. Mobile phase additives and modifiers must be selected based on the physicochemical properties of the target analytes as well as the demands of the analytical methodology employed by the researcher.

Page 7: Detection Methods in RP-HPLC for Stability Analysis

UV/Vis Detection

UV/Vis detection is a commonly used, widely applied method for the stability assessment of RP-HPLC. Its main benefit is that it can specify the wavelengths used to track degradation products. There is a stability indicating HPLC method derived for canagliflozin analysis using a photodiode array (PDA) detector at 290 nm (Azhakesan & Kuppusamy, 2023). Such procedure was specific, accurate, and precise towards the detection of canagliflozin and its decomposition products. Interestingly, this can also become a more powerful technique as it can work together with the UV/Vis spectroscopy. For example, Goblirsch et al (2023) describe a submersible sensor probe that integrates UV/Vis with fluorescence spectroscopy for the detection of biological and chemical contaminants in water (Goblirsch et all, 2023). Nurani et al. to minimize the practical challenge of overlapping peaks in the analysis of multicomponent pharmaceutical preparations (Nurani et al., 2023). Concluding Remarks: UV/Vis detection using RP-HPLC is a robust and flexible method for stability analysis. The fact that NIR is capable of selecting specific wavelengths is particularly useful to monitor the degradation products. Applying UV/Vis detection

in conjunction with other methods or data processing techniques can yield potent analytical capabilities for matrixed samples and multicomponent formulations.

Mass Spectrometry (MS) and HPLC-MS

High-performance liquid chromatography (HPLC) with mass spectrometry (MS) has been used as a key tool for analysis of stability and structural determination of degradation products. This combination of compounds has been shown to be effective in several studies. Stability analysis through HPLC-MS has high specificity and sensitivity. A stability-indicating HPLC method was established for canagliflozin with a photodiode array detector, which was able to separate the drug from its degradation products (Azhakesan & Kuppusamy, 2023). Likewise, HPLC-MS/MS with lower limits of quantification than previous GC or GC-MS methods was the analytical technique employed to detect dicofol residues in environmental water samples (Xie et al., 2023). In fact, there are some ways founded in the literature that are used to improve detection capability. To this end, a smartphone-based fluorescence detector for HPLC, which enables the simultaneous detection of fluorescent compounds with varied excitation wavelengths, was added (Shamsaei et al., 2023). In contrast to typical fluorescence detectors, this process provides a dramatically less expensive and modifiable alternative. Overall, RP-HPLC coupled with MS has several advantages for the identification of the degradation products. It has enhanced sensitivity and specificity, as well as better unknown compound detection. Moreover, the application of tandem MS (MS/MS) or high-resolution MS (HRMS) greatly boosts the availability of structural information (Mahdavijalal et al., 2024). So, effective HPLC-MS techniques have been greatly consummate for analyzing drug stability and degradation products in either pharmaceutical or environmental samples.

Other Detection Techniques

RP-HPLC provides many detection methods for stability analysis but the most common is UV-Vis spectrophotometry. In contrast, other detection approaches can offer improved sensitivity and specificity for particular analytes. The method of HPLC with fluorescence detection is able to provide relevant specificity and sensitivity for fluorescent compounds. Shamsaei et al. (2023) have developed a smartphone-based fluorescence detector for HPLC-based detection of fluorescent compounds with multiple excitation wavelengths. It offers a novel approach to co-eluted compound analysis that is economical and highly adaptable. In a different study, HPLC with fluorescence detection (HPLC-FD) was applied for the quantification of alectinib in bulk powder and urine samples (Almutairi et al., 2023). Occasionally, electrochemical detection (ECD) works well in specific settings. Wu et al. (2023) developed and validated a method using high-performance liquid chromatography with electrochemical detection (HPLC-ECD) to measure l-ascorbic acid levels in honey samples. The sensitivity is higher and the detection is faster, compared with common methods: titration, spectrophotometry, and HPLC-DAD. Nevertheless, UV-Vis detection is still the method of choice in RP-HPLC for stability studies; however, fluorescent and electrochemical detections are alternatives with higher sensitivity and specificity for specific analytes. The IXT detection method used varies depending on the specific compounds and application sensitivity required.

Page 8: Method Validation for Stability-Indicating RP-HPLC

Validation Guidelines (ICH, USP)

International Conference on Harmonization (ICH) and United States Pharmacopeia (USP) guidelines generally are followed for the development and validation of stability-indicating RP-HPLC methods employed in pharmaceutical analysis. Specificity, accuracy, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ) (Kelani et al., 2023; Siddique et al., 2023) were among key validation parameters. Various pharmaceutical compounds have been detected by stability-indicating RP-HPLC method and many such methods were developed and validated successfully. An analytical method for the simultaneous estimation of Ozenoxacin and Benzoic Acid in cream formulations has been developed, optimized, and validated as per ICH guidelines, and all validation parameters satisfied the acceptance criteria (Ramireddy & Behara, 2023). In parallel, a method was developed for simultaneous determination of eletriptan hydrobromide and itopride hydrochloride following the USP and ICH guidelines and proving to be very accurate, precise and linear (Siddique et al., 2023). Notably, a few have exceeded the conventional validation criteria. In one study, forced degradation studies were included to demonstrate the stability-indicating capability of the method (Ramireddy & Behara, 2023). A different study utilized molecular docking in order to corroborate the elution sequence of drugs and their degradation products, and they also utilized the analytical eco-scale metric in order to evaluate the environmental impact of the method (Kelani et al., 2023). Validation details play an important role in RP-HPLC stability-indicating methodology. Specificity, accuracy, precision, linearity, limit of detection (LOD), limit of quantitation (LOQ) are the main ICH and USP parameters, however, with new trends being set for the future towards more robustness and sustainability other approaches/criteria have to be introduced.

Case Studies in Method Validation

[1–11] Several case studies have reported the development and validation of stability-indicating RP-HPLC methods for pharmaceutical analyses. In 2023, Ramireddy and Behara developed and validated a stability-indicating RP-HPLC method for Ozenoxacin and Benzoic Acid in cream formulations. This method uses gradient elution on a C8 column at a 235 nm wavelength for UV detection. All validation parameters were in accordance with ICH guidelines and forced degradation studies confirmed the stability-indicating power of the method. An RP-HPLC method was validated according to the USP and ICH guidelines for Eletriptan hydrobromide and itopride hydrochloride (Siddique et al., 2023). The separation was accomplished in less than 5 min using phosphate buffer and acetonitrile as mobile phase. The validation studies included precision, accuracy, linearity, and stability studies of the combined dosage forms. An aqueous phase / HPLC method for determining canagliflozin was developed in a stability-indicating manner based on an analytical quality by design (AQbD) strategy (Azhakesan & Kuppusamy, 2023). The method employed a C18 column and UV detection at 290 nm. The stability-indicating nature of this method was supported by forced degradation studies. They confirmed this technique to be specific, precise, linear, rugged, and robust. However, several studies also utilized novel approaches for method development and validation. A green RP-HPLC has been developed for methionine and paracetamol using glycerol as a mobile phase (Habib et al., 2023). Explore the possibility of implementing such an approach as a means to enhance the environmental sustainability of the analytical method, while simultaneously still

addressing validation requirements. In conclusion, these case studies demonstrate the need for rigorous method validation practices when developing stability-indicating RP-HPLC methods for pharmaceutical applications. They showed how regulatory guidelines can be applied, explained the use of forced degradation studies, and potential innovative approaches in method development and validation.

Practical Considerations

Imparting method validation for stability-indicating RP-HPLC analysis is not free of practical consideration and challenges. Stability-indicating methods are designed to separate the active pharmaceutical ingredient (API) from its degradation products. The practice often includes forced degradation studies aimed at generating such degradants and ultimately optimizing chromatographic separation of the various components (Azhakesan & Kuppusamy, 2023; Kelani et al., 2023). Asymmetrical shifting of peaks due to repeated elution at the same temperature susceptible to viscous flow can compromise separation under conditions that give optimal separation between peaks eluting closely (Shamim et al., 2023; Siddique et al., 2023). Other essential validation parameters are linearity, accuracy, and precision, and these have to be established over an adequate range of concentration. Consistent results across the full range of concentrations, particularly at or near the limit of quantitation, can be challenging (Almutairi et al., 2023; Siddique et al., 2023). It is also necessary to perform robustness testing to make sure that small changes in the experimental conditions do not affect the performance of the method (Al-Hakkani et al., 2023; Azhakesan and Kuppusamy, 2023). The stability of the sample throughout the analysis and storage is also an important consideration. Absorption, solubility, or the breakdown of analytes over time can alter results; therefore, the stability of the solution and mobile phase must be investigated (Azhakesan & Kuppusamy, 2023; Siddique et al., 2023). For extracts from complex natural product mixtures, achieving reproducible extraction and separation of several analytes can be particularly difficult (Mohammed et al., 2023). Sensitivity can also be a limitation, especially for compounds that do not have a strong chromophore. Derivatization methods might be required to enhance the detection but will add extra complexity (Huang et al., 2023). Quantitation may experience competition from matrix effects, particular to biological or formulation samples, to which the method must be developed and validated (Almutairi et al., 2023; Carvalho et al., 2023). In conclusion, a systematic framework involving application of quality-by-design and design of experiment methods tends to alleviate these challenges and may lead to the development of a robust and reliable methods amenable to stability-indicating assays (Azhakesan & Kuppusamy, 2023; Mohammed et al., 2023; Shamim et al., 2023). The development of reliable stability-indicating RP-HPLC methods requires careful optimization of chromatographic conditions, adequate validation for all key parameters, and evaluation for method performance in representative sample matrices.

Page 9: RP-HPLC Applications in Stability Testing: Case Studies

Pharmaceutical Product Applications

RP-HPLC, have been commonly used to stability test many of the pharmaceutical products, including oral tablets, injectables, and biologics. Its application has proved to be successful in the analysis of drug stability, as shown in some case studies. Stability assessment and degradation products of active ingredients have been

identified by designing these RP-HPLC methods in oral tablets. In a recent study, a new stability-indicating HPLC method was developed and validated for the simultaneous determination of curcuminoids in Curcuma longa extracts, tablets, and capsules, along with their forced mead, degradation in different conditions (Mohammed et al., 2023). This method was specific, accurate, and precise for simultaneous quantification of the analyte mixture, as well as the analysis of degradation products. In a similar line, an HPLC method for the quantitative determination of canagliflozin in tablets was developed and validated and the stability indicating nature of the method was exercised by stressed degradation studies (Azhakesan & Kuppusamy, 2023). In-depth analysis of virological failure in the context of long-acting injectable therapy of cabotegravir and rilpivirine utilized HPLC to quantify drug plasma concentrations and assess on-treatment drug exposure (Wensing et al., 2024). The RP-HPLC application in this study can be used to derive the stability and activity of an injectable compound. Please note that the data presented retains the application of RP-HPLC concept but does not provide specific examples that illustrate the principles behind biologics in the same way elaborated in the latter part of the main data table, and which is directed towards supersaturation of singles and their impurity profiles. RP-HPLC can be used for high sensitivity and specificity of pharmaceutical products across a range of types of compounds and is helpful in stability testing. Reverse phase high performance liquid chromatography (RP-HPLC): A powerful and versatile tool for stability testing in pharmaceutical formations. This provides a definitive quantification of active ingredients and any degradation products depending on specific, precise measurement, and characterization of shelf-life final dosage forms and is thus useful to maintain active commercial product quality during shelf-life.

Monitoring Degradation Products

RP-HPLC has been extensively utilized for stability testing and tracking of degradation products for an array of pharmaceutical compounds. Few studies report its effectiveness on real-world degradation steps, such as oxidative, thermal, and photolytic conditions. (RP-HPLC) coupled with mass spectrometry was implemented in a study on Ertugliflozin to separate and detect degradation products under different stress conditions. Under any thermal, photolytic, neutral and alkaline condition, drug remained stable, whereas it degraded rapidly under acid and oxidative hydrolysis. In acid degradation we identified four new degradation products and one under oxidative conditions (Salakolusu et al., 2023). Likewise, in the case of Febuxostat, RP-HPLC-MS analysis showed detection of four degradation products upon acid hydrolysis, whereof three were newly reported (Kanagaddi et al., 2024). In some cases, however, conflicting results have been reported. While Ertugliflozin and Febuxostat were stable under the tested thermal and photolytic conditions a study on pimavanserin (PVS) showed marked degradation when subjected to the same conditions. The most severe destruction of PVS occurred through oxidative hydrolysis and thermal degradation (Nassef et al., 2024). This underscores the necessity of compound-specific stability tests. To summarize, RP-HPLC can be an effective and versatile tool for stability testing and degradation product monitoring of a wide variety of pharmaceutical compounds. With its ability to identify and characterize the degradation products of these compounds when subjected to a variety of stresses, such as oxidative, thermal, photolytic, and photochemical degradation, performed at

varying temperatures or intensities, it is an invaluable technique in both pharmaceutical research and quality control.

Regulatory Compliance

Stability testing of a drug during the approval process is required by the regulations, which is often accomplished using RP-HPLC. Multiple case studies illustrate their applications as well as their impact. 禾RP-HPLC methods for stability-indicating determinations are high in demand by regulatory bodies. One study reported development and validation of a RP-HPLC method of simultaneous estimation of Ozenoxacin and Benzoic Acid in Pharma cream formulation according to the ICH guidance. The method was determined to be stability-indicating and applicable for routine quality control analysis (Ramireddy & Behara, 2023). In analogy, another study, developed an analytical quality by design (AQbD) stability-indicating HPLC approach for the quantitation of canagliflozin. The method was validated, and it was able to assay canagliflozin in tablets and stable samples (Azhakesan & Kuppusamy, 2023). Surprisingly, researchers have already started investigating new concepts to make RP-HPLC more regulatory compliant. An example of this was a multi-attribute method (MAM) for peptide mapping liquid chromatography-mass spectrometry for batch release and stability testing under the GMP regime. It is hoped that this method will help with the technical, compliance, and regulatory factors associated with... Overall, RP-HPLC methods are crucial in fulfilling the regulatory requirements for appropriate stability testing in drug approval process. They deliver accurate, precise, stability-indicating analyses that are required for quality control and regulatory compliance. This, combined with the mainstreaming of AQbD and MAM into regulatory practice, reinforce the analytical method life cycle as part of a building block approach to product quality assurance throughout Quadrant C of the Quality by Design paradigm.

SUMMARY

This analytical technology has evolved as analytical stability-indicating RP-HPLC with wider applications in pharmaceutical quality control. Advancements in RP-HPLC strategies and their ramifications to pharmaceutical excellence, an overview. The basics in regards to stability-indicating analysis, regulatory guideline in regards to stability-studies, types of degradation observed for various drugs and the importance of stability-studies in drug development are explained. RP-HPLC provides significant advantages, including selective separation, enhanced sensitivity, and robust performance in stability analysis. The trade-off between selectivity and efficiency, however, has limited the capabilities of RP-HPLC for high-resolving power, and many challenging compounds still await resolution; consequently, recent developments in RP-HPLC columns and stationary phases (e.g., sub-2 μm particles and customized interaction sites) have greatly improved separation capabilities. With improvements in detection methods, especially mass spectrometry, and new techniques, such as fluorescent detection using smartphone chips, sensitivity and specificity have also been improved. It stresses the significance of performing method validation in accordance with ICH and USP guidelines and illustrates this with case studies of appropriately developed and validated stability-indicating RP-HPLC methods. The application of RP-HPLC in the stability testing is elaborated, where RP-HPLC is widely used for pharmaceutical product analysis, degradation product monitoring, and regulatory compliance. The review is concluded with a

summary of the major advances in RP-HPLC be it theoretical or practical for stability-indicating analysis, along with their significance with reference to pharmaceutical excellence and the highlights of the newer paradigms and future directions.

REFERENCES

1. Al-Hakkani, M. F., Ahmed, N., Hassan, M. H. A., & Abbas, A. A. (2023). Cefoperazone rapidly and sensitive quantitative assessment via a validated RP-HPLC method for different dosage forms, in-use stability, and antimicrobial activities. BMC Chemistry, 17(1). https://doi.org/10.1186/s13065-023-00989-0
2. Al-Sulaimi, S., El Jery, A., Aldrdery, M., Kushwah, R., Ashraf, G. A., & Abdullah Alsibani, M. (2023). Emerging Developments in Separation Techniques and Analysis of Chiral Pharmaceuticals. Molecules, 28(17), 6175. https://doi.org/10.3390/molecules28176175
3. Almutairi, H., Alanazi, M., Darwish, I., Bakheit, A., Darwish, H., & Alshehri, M. (2023). Development of Novel Microwell-Based Spectrofluorimetry and High-Performance Liquid Chromatography with Fluorescence Detection Methods and High Throughput for Quantitation of Alectinib in Bulk Powder and Urine Samples. Medicina, 59(3), 441. https://doi.org/10.3390/medicina59030441
4. Alperth, F., Kunert, O., Bucar, F., & Schneebauer, A. (2023). Phytochemical Analysis of Pinus cembra Heartwood-UHPLC-DAD-ESI-MSn with Focus on Flavonoids, Stilbenes, Bibenzyls and Improved HPLC Separation. Plants (Basel, Switzerland), 12(19), 3388. https://doi.org/10.3390/plants12193388
5. Amorim, A. M. B., Amorim, A. M. B., Amorim, A. M. B., Amorim, A. M. B., Amorim, A. M. B., Gaspar, A. T., Rosário-Ferreira, N., Piochi, L. F., Piochi, L. F., Piochi, L. F., Preto, A. J., Moreira, I. S., Preto, A. J., Rosário-Ferreira, N., Preto, A. J., Moreira, I. S., Gaspar, A. T., Moreira, I. S., & Gaspar, A. T. (2024). Advancing Drug Safety in Drug Development: Bridging Computational Predictions for Enhanced Toxicity Prediction. Chemical Research in Toxicology, 37(6), 827–849. https://doi.org/10.1021/acs.chemrestox.3c00352
6. Axente, R. E., Vlasceanu, A.-M., Nitescu, V. G., Baconi, D. L., Stan, M., & Chitescu, C. L. (2023). Application of Ionic Liquids as Mobile Phase Additives for Simultaneous Analysis of Nicotine and Its Metabolite Cotinine in Human Plasma by HPLC–DAD. Molecules, 28(4), 1563. https://doi.org/10.3390/molecules28041563
7. Azhakesan, A., & Kuppusamy, S. (2023). Analytical Quality by Design-Assisted HPLC Method for Quantification of Canagliflozin and Stability Studies. ACS Omega, 8(8), 7407–7414. https://doi.org/10.1021/acsomega.2c06038
8. Basak, S., Shaik, L., & Chakraborty, S. (2023). Effect of ultraviolet and pulsed light treatments on ascorbic acid content in fruit juices-A review of the degradation mechanism. Food Chemistry Advances, 2, 100333. https://doi.org/10.1016/j.focha.2023.100333
9. Bonciarelli, S., Desantis, J., Goracci, L., & Cerquiglini, S. (2023). MassChemSite for In-Depth Forced Degradation Analysis of PARP Inhibitors Olaparib, Rucaparib, and Niraparib. ACS Omega, 8(7), 7005–7016. https://doi.org/10.1021/acsomega.2c07815

10. Deschamps, E., Calabrese, V., Schmitz, I., Hubert-Roux, M., Castagnos, D., & Afonso, C. (2023). Advances in Ultra-High-Resolution Mass Spectrometry for Pharmaceutical Analysis. Molecules (Basel, Switzerland), 28(5), 2061. https://doi.org/10.3390/molecules28052061
11. Dobó, M., Köteles, I., Fiser, B., Kis, C., Szabó, Z.-I., Dombi, G., Fiser, B., Tóth, G., Köteles, I., Szabó, Z.-I., & Fiser, B. (2024). Simultaneous Determination of Enantiomeric Purity and Organic Impurities of Dexketoprofen Using Reversed-Phase Liquid Chromatography-Enhancing Enantioselectivity through Hysteretic Behavior and Temperature-Dependent Enantiomer Elution Order Reversal on Polysaccharide Chiral Stationary Phases. International Journal of Molecular Sciences, 25(5), 2697. https://doi.org/10.3390/ijms25052697
12. Elsheikh, S. G., El-Mosallamy, S. S., Fayez, Y. M., & Hassan, A. M. E. (2023). Green analytical chemistry and experimental design: a combined approach for the analysis of zonisamide. BMC Chemistry, 17(1). https://doi.org/10.1186/s13065-023-00942-1
13. Fernandez Lynch, H., Sachs, R. E., Herder, M., Ramachandran, R., Ross, J. S., & Lee, S. (2023). Extending the US Food and Drug Administration's Postmarket Authorities. JAMA Health Forum, 4(6), e231313. https://doi.org/10.1001/jamahealthforum.2023.1313
14. Gamal Fawzy, M., & Kamel, E. B. (2023). Sustainable Stability-Indicating spectra manipulations for the concurrent quantification of a novel Anti-COVID-19 drug and its active Metabolite: Green profile assessment. Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy, 300, 122911. https://doi.org/10.1016/j.saa.2023.122911
15. Gervais, A., Dirksen, E. H. C., Pohl, T., Bechtold-Peters, K., Burkitt, W., D'Alessio, V., Greven, S., Lennard, A., Li, X., Lössner, C., Niu, B., Reusch, D., O'Riordan, T., Shearer, J. W., Spencer, D., Xu, W., & Yi, L. (2023). Compliance and regulatory considerations for the implementation of the multi-attribute-method by mass spectrometry in a quality control laboratory. European Journal of Pharmaceutics and Biopharmaceutics, 191, 57–67. https://doi.org/10.1016/j.ejpb.2023.08.008
16. Goblirsch, T., Penzel, S., Rudolph, M., Borsdorf, H., & Mayer, T. (2023). In Situ Water Quality Monitoring Using an Optical Multiparameter Sensor Probe. Sensors, 23(23), 9545. https://doi.org/10.3390/s23239545
17. Gong, Y.-N., Liang, R.-X., Zhang, Y.-P., Wang, B.-J., Wang, Y., Zhang, J.-H., Xie, S.-M., Yuan, L.-M., & Ma, Q.-Y. (2023). Preparation of Chiral Porous Organic Cage Clicked Chiral Stationary Phase for HPLC Enantioseparation. Molecules, 28(7), 3235. https://doi.org/10.3390/molecules28073235
18. Gray, M., Tong, W., Xu, J., & Wu, L. (2023). Classifying Free Texts into Predefined Sections Using AI in Regulatory Documents: A Case Study with Drug Labeling Documents. Chemical Research in Toxicology, 36(8), 1290–1299. https://doi.org/10.1021/acs.chemrestox.3c00028
19. Gumieniczek, A., & Berecka-Rycerz, A. (2023). Metabolism and Chemical Degradation of New Antidiabetic Drugs: A Review of Analytical Approaches for Analysis of Glutides and Gliflozins. Biomedicines, 11(8), 2127. https://doi.org/10.3390/biomedicines11082127

20. Guo, J., Kufer, R., Li, D., Wohlrab, S., Greenwood-Goodwin, M., & Yang, F. (2023). Technical advancement and practical considerations of LC-MS/MS-based methods for host cell protein identification and quantitation to support process development. MAbs, 15(1). https://doi.org/10.1080/19420862.2023.2213365
21. Haas, C. P., Nicholls, R., Mcdonald, M. A., Niedenführ, S., Jin, E. H., Di Rocco, L., Koscher, B. A., Leweke, S., Greeves, E., Guimond, N., Hillenbrand, J., Kayser, H., Lübbesmeyer, M., Barber, D. M., Jensen, K. F., & Volpin, G. (2023). Open-Source Chromatographic Data Analysis for Reaction Optimization and Screening. ACS Central Science, 9(2), 307–317. https://doi.org/10.1021/acscentsci.2c01042
22. Habib, A., Mabrouk, M. M., Fekry, M., & Mansour, F. R. (2023). Glycerol-Based Green RP-HPLC Method for Simultaneous Determination of Methionine and Paracetamol in Pharmaceutical Tablets. Chromatographia, 86(11–12), 707–716. https://doi.org/10.1007/s10337-023-04283-y
23. Haq, N., Aloatibi, F. O., Shakeel, F., Alam, P., Alshehri, S., Asdaq, S. M. B., & Ghoneim, M. M. (2023). Determination of Pterostilbene in Pharmaceutical Products Using a New HPLC Method and Its Application to Solubility and Stability Samples. Separations, 10(3), 178. https://doi.org/10.3390/separations10030178
24. Hausladen, D. M., & Peña, J. (2023). Organic buffers act as reductants of abiotic and biogenic manganese oxides. Scientific Reports, 13(1). https://doi.org/10.1038/s41598-023-32691-5
25. He, X. Z., Powers, T. W., Huang, S., Liu, Z., Shi, H., Orlet, J. D., Mo, J. J., Srinivasan, S., Jacobs, S., Zhang, K., Runnels, H. A., Anderson, M. M., & Lerch, T. F. (2023). Development of an icIEF assay for monitoring AAV capsid proteins and application to gene therapy products. Molecular Therapy - Methods & Clinical Development, 29, 133–144. https://doi.org/10.1016/j.omtm.2023.03.002
26. Huang, X.-F., Xue, Y., Yong, L., Wang, T.-T., Luo, P., & Qing, L.-S. (2023). Chemical derivatization strategies for enhancing the HPLC analytical performance of natural active triterpenoids. Journal of Pharmaceutical Analysis, 14(3), 295–307. https://doi.org/10.1016/j.jpha.2023.07.004
27. Hussain, A., Ramzan, M., Khuroo, T., Altamimi, M. A., Alnemer, O. A., & Mohd Siddique, M. U. (2023). HSPiP, Computational Modeling, and QbD-Assisted Optimized Method Validation of 5-Fluorouracil for Transdermal Products. ACS Omega, 9(1), 903–916. https://doi.org/10.1021/acsomega.3c07061
28. Jain, S., & Shah, R. P. (2023). Drug-Excipient Compatibility Study Through a Novel Vial-in-Vial Experimental Setup: A Benchmark Study. AAPS Pharm SciTech, 24(5). https://doi.org/10.1208/s12249-023-02573-0
29. Jeelani, S., & Kouznetsova, N. (2023). A new stability-indicating HPLC-UV method for determination of amlodipine besylate and its impurities in drug substance. Heliyon, 9(9), e19993. https://doi.org/10.1016/j.heliyon.2023.e19993
30. Kanagaddi, R., Kaliyaperumal, M., Salakolusu, S., Katari, N. K., Chintala, V., Ranga, M., Murty, J., & Nannapaneni, S. S. (2024). Isolation and identification of forced degradation products of Febuxostat. SEPARATION SCIENCE PLUS, 7(5). https://doi.org/10.1002/sscp.202300237
31. Kelani, K. M., Fayez, Y. M., Abdel-Raoof, A. M., Fekry, R. A., & Hassan, S. A. (2023). Development of an eco-friendly HPLC method

for the stability indicating assay of binary mixture of ibuprofen and phenylephrine. BMC Chemistry, 17(1). https://doi.org/10.1186/s13065-023-01056-4

32. Khramtsov, P., Kropaneva, M., Galaeva, Z., Rayev, M., Mukhlynina, E., & Minin, A. (2023). Optimizing the Composition of the Substrate Enhances the Performance of Peroxidase-like Nanozymes in Colorimetric Assays: A Case Study of Prussian Blue and 3,3'-Diaminobenzidine. Molecules, 28(22), 7622. https://doi.org/10.3390/molecules28227622
33. Kinoshita, S., Iharada, T., Kobayashi, Y., Tsukagoshi, K., Sakamaki, H., Ishikawa, D., & Obata, Y. (2023). Development of a HPLC system using a phase-separation multiphase flow as an eluent coupled to a silica-particle packed column. Analytical Sciences: The International Journal of the Japan Society for Analytical Chemistry, 39(9), 1483–1491. https://doi.org/10.1007/s44211-023-00363-z
34. Mahdavijalal, M., Protti, M., Petio, C., Mandrioli, R., & Staffilano, G. (2024). Innovative Solid-Phase Extraction Strategies for Improving the Advanced Chromatographic Determination of Drugs in Challenging Biological Samples. Molecules, 29(10), 2278. https://doi.org/10.3390/molecules29102278
35. Mallik, A. K., Schmitz, O. J., Montero, L., Rösler, J., & Meckelmann, S. W. (2023). Surface Modification of Silica with β-Alanine Derivatives for Unique Applications in Liquid Chromatography. ACS Applied Materials & Interfaces, 15(46), 54176–54184. https://doi.org/10.1021/acsami.3c11932
36. Menda, J., Kowtharapu, L. P., Jonnalagadda, S. B., Kanuparthy, P. R., Chintala, V., & Katari, N. K. (2024). Quality by Design Tool Assessed Ultraperformance Liquid Chromatography Method for the Analysis of Remogliflozin and Teneligliptin in Oral Dosage Form. ACS Omega, 9(11). https://doi.org/10.1021/acsomega.3c04589
37. Mohammed, H. A., Ahmed, A. M., Khan, R. A., Hegazy, A. M., & Alsahabi, D. S. (2023). Analytical Purity Determinations of Universal Food-Spice Curcuma longa through a QbD Validated HPLC Approach with Critical Parametric Predictors and Operable-Design's Monte Carlo Simulations: Analysis of Extracts, Forced-Degradants, and Capsules and Tablets-Based Pharmaceutical Dosage Forms. Foods, 12(5), 1010. https://doi.org/10.3390/foods12051010
38. Najmi, A., Rehman, Z. U., Zoghebi, K., Alhazmi, H. A., Albratty, M. M., Haroobi, Q. Y. H., Sayram, I. M. A., Saleh, M. A., Qaser, W. M. A., & Qaysi, A. A. H. (2023). Central composite design (CCD) approach to develop HPLC method for caffeine: Application to coffee samples analysis of Jazan region, Saudi Arabia. Journal of Saudi Chemical Society, 28(1), 101772. https://doi.org/10.1016/j.jscs.2023.101772
39. Nassef, H. M., Mahgoub, S. M., El-Atawy, M. A., Mohamed, M. A., Bashal, A. H., Ahmed, H. A., & Alanazi, T. Y. A. (2024). A novel Six Sigma approach and eco-friendly RP-HPLC technique for determination of pimavanserin and its degraded products: Application of Box–Behnken design. Reviews in Analytical Chemistry, 43(1). https://doi.org/10.1515/revac-2023-0073
40. Nekkalapudi, A. R., Srinivasu, N., Kumar, C. V., Katari, N. K., Gumudavelli, S., & Pippalla, S. (2023). A validated stability‐indicating reversed‐phase liquid chromatography method for the quantification of Ixabepilone (Oncology drug) in the parenteral dosage form. SEPARATION SCIENCE PLUS, 6(4), 2200099. https://doi.org/10.1002/sscp.202200099

41. Niezen, L. E., Bos, T. S., Schoenmakers, P. J., Somsen, G. W., & Pirok, B. W. J. (2023). Capacitively coupled contactless conductivity detection to account for system-induced gradient deformation in liquid chromatography. Analytica Chimica Acta, 1271, 341466. https://doi.org/10.1016/j.aca.2023.341466
42. Nurani, L. H., Edityaningrum, C. A., Windarsih, A., Rohman, A., Putri, A. R., Irnawati, I., & Guntarti, A. (2023). Chemometrics-Assisted UV-Vis Spectrophotometry for Quality Control of Pharmaceuticals: A Review. Indonesian Journal of Chemistry, 23(2), 542. https://doi.org/10.22146/ijc.74329
43. Queral-Beltran, A., Marín-García, M., Lacorte, S., & Tauler, R. (2023). UV-Vis absorption spectrophotometry and LC-DAD-MS-ESI (+)-ESI (−) coupled to chemometrics analysis of the monitoring of sulfamethoxazole degradation by chlorination, photodegradation, and chlorination/photodegradation. Analytica Chimica Acta, 1276, 341563. https://doi.org/10.1016/j.aca.2023.341563
44. Ramireddy, A. R., & Behara, D. K. (2023). Analytical method validation on simultaneous estimation of Ozenoxacin and Benzoic acid in pharmaceutical formulation. Acta Chromatographica, 35(3), 278–285. https://doi.org/10.1556/1326.2022.01064
45. Rathee, A., Verma, S., Kohli, K., Aodah, A., Ansari, M. J., Vohora, D., Sultana, Y., & Solanki, P. (2023). Simultaneous Determination of Posaconazole and Hemp Seed Oil in Nanomicelles through RP-HPLC via a Quality-by-Design Approach. ACS Omega, 8(33), 30057–30067. https://doi.org/10.1021/acsomega.3c02097
46. Salakolusu, S., Jonnalagadda, S. B., Sharma, G. V. R., Kaliyaperumal, M., Katari, N. K., Puppala, U., & Ranga, M. (2023). Identification, isolation, and structural characterization of novel forced degradation products of Ertugliflozin using advanced analytical techniques. Scientific Reports, 13(1). https://doi.org/10.1038/s41598-023-36289-9
47. Samara, C., Desmeules, J., Henry, C., Pinçon, A., Garcia, A., Vallotton, L., & Cariolato, L. (2023). Safety Surveillance During Drug Development: Comparative Evaluation of Existing Regulations. Advances in Therapy, 40(5), 2147–2185. https://doi.org/10.1007/s12325-023-02492-3
48. Shamim, A., Iqbal, M., Ansari, M. J., Aqil, M., Aodah, A., Mirza, M. A., Ali, A., & Iqbal, Z. (2023). QbD-Engineered Development and Validation of a RP-HPLC Method for Simultaneous Estimation of Rutin and Ciprofloxacin HCl in Bilosomal Nanoformulation. ACS Omega, 8(24), 21618–21627. https://doi.org/10.1021/acsomega.3c00956
49. Shamsaei, D., Hsieh, S.-A., Ocaña-Rios, I., Ryan, S. J., & Anderson, J. L. (2023). Smartphone as a fluorescence detector for high-performance liquid chromatography. Analytica Chimica Acta, 1280, 341863. https://doi.org/10.1016/j.aca.2023.341863
50. Siddique, W., Khan, R., Zaman, M., Malik, T., Saif, A., Alshamrani, M., Sabei, F. Y., Asghar, F., Gul, M., Shamim, Q.-U.-A., Sarfraz, R. M., Salawi, A., & Almoshari, Y. (2023). Method development and validation for simultaneous determination of Eletriptan hydrobromide and Itopride hydrochloride from fast dissolving buccal films by using RP-HPLC. Acta Chromatographica, 35(4), 310–318. https://doi.org/10.1556/1326.2022.01072
51. Sun, Y., Ohkouchi, N., Ogawa, N. O., Takano, Y., Ishikawa, N. F., & Blattmann, T. M. (2023). Application of a porous graphitic

carbon column to carbon and nitrogen isotope analysis of underivatized individual amino acids using high-performance liquid chromatography coupled with elemental analyzer/isotope ratio mass spectrometry. Rapid Communications in Mass Spectrometry, 37(17). https://doi.org/10.1002/rcm.9602

52. Taghavi, T., Patel, H., & Rafie, R. (2023). Extraction Solvents Affect Anthocyanin Yield, Color, and Profile of Strawberries. Plants, 12(9), 1833. https://doi.org/10.3390/plants12091833
53. Ventouri, I. K., Veelders, S., Passamonti, M., Endres, P., Roemling, R., Schoenmakers, P. J., Somsen, G. W., Haselberg, R., & Gargano, A. F. G. (2023). Micro-flow size-exclusion chromatography for enhanced native mass spectrometry of proteins and protein complexes. Analytica Chimica Acta, 1266, 341324. https://doi.org/10.1016/j.aca.2023.341324
54. Weber, J., Buske, J., Mäder, K., Garidel, P., & Diederichs, T. (2023). Oxidation of polysorbates – An underestimated degradation pathway? International Journal of Pharmaceutics: X, 6, 100202. https://doi.org/10.1016/j.ijpx.2023.100202
55. Wensing, A. M. J., Wensing, A. M. J., Van Welzen, B. J., Van Nieuwkoop, C., Burger, D., Ter Beest, G., Van Twillert, G., Prins, J. M., Gisolf, J. H., Van Der Valk, M., Van Der Valk, M., Van Lelyveld, S. F. L., & Geerlings, S. E. (2024). Virological Failure After Switch to Long-Acting Cabotegravir and Rilpivirine Injectable Therapy: An In-depth Analysis. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 79(1), 189–195. https://doi.org/10.1093/cid/ciae016
56. Williams, M. L., Gionfriddo, E., Godage, N. H., Olomukoro, A. A., & Emmons, R. V. (2023). Matrix effects demystified: Strategies for resolving challenges in analytical separations of complex samples. Journal of Separation Science, 46(23). https://doi.org/10.1002/jssc.202300571
57. Wu, F., Cao, W., Liu, W., Chen, S., Xu, F., Luo, H., Zhao, H., & Cheng, N. (2023). A High-Performance Liquid Chromatography with Electrochemical Detection Method Developed for the Sensitive Determination of Ascorbic Acid: Validation, Application, and Comparison with Titration, Spectrophotometric, and High-Performance Liquid Chromatography with Diode-Array Detection Methods. Foods, 12(16), 3100. https://doi.org/10.3390/foods12163100
58. Wu, W. W., Vicente, J., Bende, G., Johannesen, L., Garnett, C., Strauss, D. G., Choe, M., & Stockbridge, N. L. (2023). ICH S7B In Vitro Assays Do Not Address Mechanisms of QTC Prolongation for Peptides and Proteins - Data in Support of Not Needing Dedicated QTC Studies. Clinical Pharmacology & Therapeutics, 114(6), 1332–1341. https://doi.org/10.1002/cpt.3047
59. Xiang, W., Xu, F., Wan, D., Wang, X., Luo, F., & Chen, Y. (2023). Mechanistic investigation of direct photodegradation of chloroquine phosphate under simulated sunlight. Chemosphere, 335, 139093. https://doi.org/10.1016/j.chemosphere.2023.139093
60. Xie, J., Ma, Y., Zhu, L., Liu, X., Guo, Y., Mao, L., Jiang, H., Zhang, L., & Zheng, Y. (2023). Spontaneous In-Source Fragmentation Reaction Mechanism and Highly Sensitive Analysis of Dicofol by Electrospray Ionization Mass Spectrometry. Molecules, 28(9), 3765. https://doi.org/10.3390/molecules28093765
61. Zheng, G., Cheng, Y., & Su, H. (2023). Role of Carbon Dioxide, Ammonia, and Organic Acids in Buffering Atmospheric Acidity: The Distinct Contribution in Clouds and Aerosols.

Environmental Science & Technology, 57(34), 12571–12582. https://doi.org/10.1021/acs.est.2c09851.

HOW TO CITE: Harshad Tanpure*, Dr. Vishwas Bahgat, Dr. Deepak Kardile, Dr. Rajkumar Shete, M. M. Karne, Revolutionizing Stability-Indicating Analysis: Advanced RP-HPLC Strategies for Pharmaceutical Excellence, Int. J. of Pharm. Sci., 2025, Vol 3, Issue 5, 4466-4489. https://doi.org/10.5281/zenodo.15522235