



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



Review Article

Combination Drug Analysis: Challenges and Approaches in HPLC Method Development

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ARTICLE INFO

Published: 09 June 2025

Keywords:

Combination drugs, HPLC, Analytical challenges, Quality by Design, Method validation, Hyphenated techniques, green chemistry, Automation

DOI:

10.5281/zenodo.15624782

ABSTRACT

Combination drug therapy has emerged as a pivotal strategy in the management of chronic and multifactorial diseases, offering synergistic therapeutic effects, enhanced patient adherence, and a reduced risk of adverse reactions. High-Performance Liquid Chromatography (HPLC) remains a critical analytical tool for the precise qualitative and quantitative evaluation of such formulations, owing to its high sensitivity, selectivity, and reliability. Nevertheless, the concurrent analysis of multiple active pharmaceutical ingredients (APIs) presents notable challenges, including co-elution, varying physicochemical properties, matrix complexities, and stability-related concerns. Regulatory frameworks demand the implementation of thoroughly validated and scientifically sound analytical methods to guarantee product safety, efficacy, and quality. This review comprehensively addresses the methodological challenges encountered in HPLC analysis of combination drugs and examines advanced strategies such as Quality by Design (QbD), innovative detection technologies, and refined sample preparation techniques to optimize analytical outcomes. In addition, this review features selected case studies that illustrate the practical implementation of HPLC methodologies in the analysis of complex combination drug regimens, including antihypertensive, antidiabetic, and antimicrobial therapies. It further examines emerging technological advancements poised to transform analytical practices—such as the integration of hyphenated techniques (e.g., LC-MS/MS, UHPLC), automation systems, artificial intelligence-driven data processing, and eco-friendly (green) analytical approaches. Collectively, these innovations signify a paradigm shift in the analytical landscape, steering towards more efficient, sustainable, and intelligent method development. This comprehensive analysis serves as a critical reference for pharmaceutical analysts, research scientists, and regulatory authorities engaged in the rigorous development, validation, and optimization of HPLC methods for combination drug products.

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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.



INTRODUCTION

Importance of Combination Therapy

The advent of combination drug therapy has significantly transformed therapeutic strategies for numerous diseases, particularly chronic and multifactorial conditions such as HIV/AIDS, diabetes mellitus, hypertension, and tuberculosis [1,2]. This approach involves the simultaneous administration of two or more pharmacological agents, each targeting distinct molecular pathways or mechanisms of action, with the aim of achieving synergistic or additive therapeutic benefits and optimizing clinical outcomes [3]. For instance, in the management of hypertension, combination regimens involving calcium channel blockers and angiotensin receptor blockers have demonstrated superior efficacy in blood pressure control and a reduced incidence of adverse effects compared to monotherapy [4]. The growing adoption of combination drug therapies across global pharmaceutical markets is largely attributed to their ability to improve patient adherence by

reducing pill burden, mitigating the risk of drug resistance—particularly in the treatment of infectious diseases—and allowing for more flexible dose adjustments [5,6]. Moreover, fixed-dose combinations (FDCs) significantly enhance therapeutic outcomes and minimize the likelihood of medication errors, which are especially critical in the context of complex treatment protocols and long-term disease management [7]. Given the inherent complexity of combination drug products, stringent quality control measures and highly reliable analytical techniques are essential to ensure batch-to-batch consistency, potency, purity, and long-term stability throughout the product's shelf life. To meet these demands, analytical methodologies must possess the capability to accurately identify and quantify multiple active pharmaceutical ingredients (APIs) simultaneously within complex formulation matrices. These challenges have catalyzed extensive research efforts aimed at developing robust, sensitive, and efficient analytical protocols and procedures [8].

Figure 1: Simulated HPLC Chromatogram

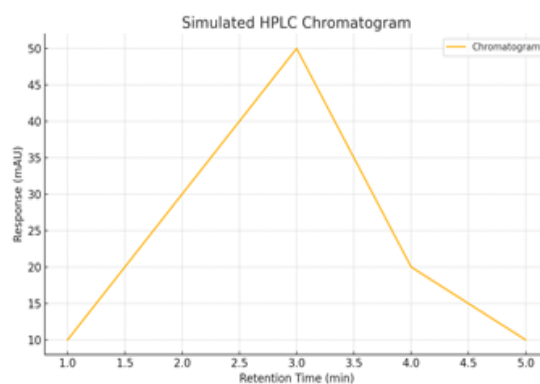


Figure 1 shows a simulated chromatogram representing typical retention peaks of a combination drug analysis.

Role of HPLC in Combination Drug Analysis

High-Performance Liquid Chromatography (HPLC) remains the analytical technique of choice for the evaluation of combination drug

formulations, owing to its exceptional adaptability, high sensitivity, and capacity for simultaneous multi-analyte quantification [9]. Through systematic modification of



chromatographic parameters—including stationary phase selection, mobile phase composition, flow rate optimization, and detection modalities—HPLC offers high-resolution separation and reproducible results, making it versatile enough to accommodate a broad spectrum of chemically diverse compounds^[10]. When coupled with advanced detection systems such as UV-Visible spectroscopy, Photodiode Array (PDA), Fluorescence, and Mass Spectrometry (MS), HPLC demonstrates a remarkable ability to resolve analytes with closely related physicochemical properties. This enables comprehensive profiling of active pharmaceutical ingredients (APIs), impurities, and degradation products within a single analytical run. As a result, HPLC has become an indispensable tool in pharmaceutical quality control laboratories and research settings, where precision, reliability, and regulatory compliance are paramount^[11,12]. Moreover, HPLC plays a crucial role in bioanalytical assays, stability assessments, and dissolution testing—key components in drug development, formulation refinement, and regulatory approval processes. Its inherent versatility in method development facilitates adherence to stringent regulatory requirements specific to combination drug products, ensuring robust and compliant analytical evaluation throughout the product lifecycle^[13].

Overview of Challenges in Analytical Method Development for Combination Drugs

Method development for combination drug products presents unique and complex challenges that surpass those encountered in single-drug analyses. Central to these difficulties is the necessity to simultaneously separate multiple active pharmaceutical ingredients (APIs), which often exhibit considerable variation in physicochemical properties such as polarity,

molecular weight, and ionization characteristics^[14]. Consequently, chromatographic conditions optimized for one analyte may prove suboptimal or even detrimental for others, complicating the development of a unified, efficient separation method^[15]. A prevalent challenge in the chromatographic analysis of combination drugs is co-elution, or peak overlap, where two or more compounds elute at similar retention times, potentially leading to inaccurate identification and quantification^[16]. This issue is further compounded by matrix interferences arising from formulation excipients, degradation products, and endogenous substances, which complicate analyte detection and necessitate the implementation of sophisticated sample preparation protocols and selective detection strategies^[17]. The inherent instability of certain APIs under standard chromatographic conditions, which leads to the formation of degradation products that may interfere with analyte peaks, introduces additional complexity to drug stability assessment during analysis and storage. Consequently, the development of stability-indicating analytical methods capable of unequivocally differentiating intact APIs from their degradation products is imperative^[18]. Moreover, to comply with stringent regulatory validation criteria established by agencies such as the European Medicines Agency (EMA), the U.S. Food and Drug Administration (FDA), and the International Council for Harmonisation (ICH), analytical methods must demonstrate specificity, linearity, accuracy, precision, robustness, and sensitivity for each constituent analyte within the combination drug product. Achieving these comprehensive requirements across multiple APIs necessitates a systematic, iterative, and carefully optimized method development process^[19,20].

1.4 Regulatory Significance



To ensure the therapeutic safety, efficacy, and quality of combination drug products, regulatory frameworks impose stringent requirements. Regulatory authorities emphasize the need for rigorous analytical method validation to guarantee the accurate and consistent quantification of each active pharmaceutical ingredient (API) throughout the product's lifecycle ^[21]. Additionally, compliance with pharmacopeial standards and regulatory guidelines mandates that analytical methods be capable of reliably detecting impurities and degradation products within established acceptance criteria ^[22]. The ICH Q2(R1) guideline delineates essential validation parameters for analytical methods employed in combination drug products, encompassing specificity, linearity, accuracy, precision, detection limit, quantitation limit, range, and robustness. Furthermore, stability-indicating methods—which are integral components of regulatory submissions and product dossiers—play a pivotal role in detecting and characterizing changes arising during manufacturing, handling, and storage ^[23]. Given the complexity of combination drug formulations, analytical strategies must prioritize method robustness and regulatory compliance to mitigate approval risks, thereby facilitating smoother regulatory review and expedited market authorization ^[24].

2. Challenges in HPLC Method Development for Combination Drugs

2.1 Co-elution of Compounds

A major analytical challenge in the HPLC analysis of combination drug products is co-elution. When multiple APIs exhibit similar retention behaviours due to comparable polarity, molecular structure, or interactions with the stationary phase, they may elute simultaneously or partially overlap, producing broad, unresolved peaks ^[25]. Such peak overlap complicates accurate quantification and

can lead to inaccurate assay results. For example, structural similarities between valsartan and amlodipine in antihypertensive combination formulations result in closely eluting peaks, necessitating meticulous optimization of chromatographic conditions to achieve baseline separation ^[26]. Strategies to mitigate co-elution include employing alternative stationary phases such as phenyl or cyano columns instead of conventional C18, adjusting mobile phase pH and composition, and implementing gradient elution techniques ^[27]. Additionally, advanced column technologies—such as ultra-high-performance liquid chromatography (UHPLC) columns and core-shell particle packing—enhance separation efficiency and peak capacity, thereby facilitating superior resolution of complex mixtures ^[28].

2.2 Varying Physicochemical Properties

Combination drug formulations often involve APIs with markedly diverse physicochemical properties, including polarity, ionization behavior, solubility, and chemical stability. A prime example is the combination of hydrophilic metformin and lipophilic glimepiride, which exhibit fundamentally different retention mechanisms and require distinct chromatographic conditions for optimal separation. Developing a single method that accommodates such disparities necessitates extensive screening and optimization of both mobile and stationary phases to achieve satisfactory resolution and analytical performance ^[29]. pH control is a critical parameter in the chromatographic separation of ionizable compounds, as the degree of ionization significantly influences retention behavior and peak morphology. The choice and concentration of buffer systems must be carefully aligned with the column chemistry and detection method to preserve analyte stability and ensure consistent performance during analysis ^[30]. Additionally,



the incorporation of mobile phase modifiers—such as surfactants or ion-pairing agents—can enhance retention and selectivity for challenging analytes ^[31]. Optimization of gradient elution profiles and solvent strength is also essential to ensure complete elution of all components within an acceptable runtime, without compromising chromatographic resolution ^[32].

2.3 Matrix Interference

Pharmaceutical formulations often present complex sample matrices comprising excipients, contaminants, and degradation products, which may co-elute with target analytes or interfere with detection signals ^[33]. In bioanalytical applications, biological matrices such as plasma or urine introduce additional challenges due to the presence of endogenous substances that contribute to matrix-related interferences ^[34]. These matrix effects can compromise method sensitivity and accuracy—manifesting as baseline noise in UV/PDA detection or as ion suppression/enhancement in LC-MS analyses ^[35]. To mitigate such effects, sample cleanup techniques like solid-phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation are commonly employed, albeit at the cost of increased analytical time and operational complexity ^[36]. Thus, rigorous optimization of chromatographic conditions is essential to effectively resolve analytes from matrix constituents. In some cases, the implementation of highly selective detection methods—such as fluorescence or tandem mass spectrometry—becomes necessary to achieve the required analytical specificity ^[37].

2.4 Stability Issues

The reliability of an analytical method is significantly influenced by the chemical and physical stability of active pharmaceutical

ingredients (APIs) throughout sample preparation, storage, and chromatographic analysis. Exposure to stress factors such as heat, light, moisture, extreme pH conditions, or oxidative environments can lead to degradation, potentially generating by-products that co-elute with APIs or interfere with their detection ^[38]. To elucidate degradation pathways and identify potential impurities, forced degradation studies are conducted under simulated stress conditions. These studies are essential for the development of robust stability-indicating methods capable of differentiating APIs from their degradation products ^[39,40]. Chromatographic techniques employed in this context must possess high sensitivity and selectivity to ensure accurate profiling of both parent compounds and related degradants. Maintaining analyte integrity may require the use of stabilizing agents, antioxidants, or optimized storage conditions tailored to the physicochemical properties of the analyte ^[41].

2.5 Regulatory and Validation Challenges

The validation of analytical methods for combination drug products is inherently more complex than for single-API formulations, due to the requirement to simultaneously establish performance criteria for multiple constituents [19]. Regulatory guidelines mandate comprehensive validation parameters, including specificity for each API and its potential impurities, linearity across the required concentration ranges, intra- and inter-day precision, accuracy, and robustness under varied analytical conditions ^[42]. Additionally, system suitability tests must be rigorously defined for each analyte peak, incorporating critical chromatographic parameters such as theoretical plate count, tailing factor, and resolution. These stringent expectations necessitate meticulous method design and optimization, significantly increasing the



development workload and minimizing the risk of revalidation during regulatory review ^[43].

3. Approaches and Strategies for HPLC Method Development in Combination Drugs

3.1 Selection of Chromatographic Conditions

Method development for combination drug analysis begins with the strategic selection of appropriate stationary and mobile phases. While reversed-phase chromatography (RPC) using C18 columns is widely adopted due to its versatility, certain analyte combinations benefit from alternative stationary phases such as phenyl-hexyl, cyano, or polar-embedded columns, which can offer improved selectivity and resolution for structurally diverse APIs ^[44].

Table 1. summarizes various HPLC column types and their suitability for different analyte properties.

Table 1: Comparison of Common HPLC Columns		
Column Type	Polarity Suitability	Typical Applications
C18	Non-polar	General purpose for non-polar compounds
Phenyl	Moderately polar	Aromatic compounds, π - π interactions
Cyano	Polar	Compounds with polar functional groups
Ion-exchange	Ionic	Charged analytes (acids/bases)

The mobile phase typically comprises an organic modifier—commonly methanol or acetonitrile—combined with an aqueous buffer. Fine-tuning the pH and ionic strength of the buffer is essential for optimizing analyte ionization, minimizing peak tailing, and enhancing chromatographic performance ^[45]. For complex formulations where analytes exhibit markedly different retention behaviors, gradient elution is preferred, as it facilitates the efficient elution of both polar and hydrophobic components within a practical

analysis timeframe ^[46]. Moreover, column temperature modulation can significantly influence analyte-stationary phase interactions, thereby improving peak symmetry and resolution. Systematic method scouting—through structured design-of-experiment (DoE) or screening trials—streamlines the identification of optimal chromatographic conditions ^[47].

3.2 Quality by Design (QbD)

To move beyond traditional trial-and-error practices, **Quality by Design (QbD)** introduces a systematic, science-driven, and risk-based approach to analytical method development ^[48]. Central to this framework is the establishment of an **Analytical Target Profile (ATP)**, which defines the intended method performance criteria from the outset and aligns all developmental activities with regulatory and product quality expectations. Utilizing Design of Experiments (DoE) enables the simultaneous evaluation of multiple critical method parameters—such as mobile phase pH, organic solvent composition, flow rate, and column temperature. This multivariate analysis facilitates the identification of parameter interactions and their collective influence on method attributes such as resolution, retention time, and peak symmetry ^[49]. By promoting robust method design, enhancing analytical understanding, and minimizing variability, QbD significantly reduces method development timelines while improving reliability. Moreover, its alignment with regulatory expectations ensures enhanced compliance, process transparency, and method lifecycle management under frameworks such as ICH Q8(R2), Q9, and Q14 ^[50].

3.3 Advanced Detection Techniques

The analytical power of HPLC is significantly enhanced through integration with advanced



detection systems, enabling precise characterization of complex combination drug formulations. Photodiode Array (PDA) detectors allow for full-spectrum acquisition, facilitating peak purity assessment and differentiation of co-eluting compounds based on their unique UV absorbance profiles [51]. For analytes with intrinsic fluorescence or those derivatized with fluorescent tags, fluorescence detectors provide superior sensitivity and selectivity, particularly useful in trace-level quantification [52]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) stands at the forefront of modern detection techniques, offering exceptional specificity and sensitivity, even within complex biological matrices such as plasma or tissue homogenates [53]. The use of Multiple Reaction Monitoring (MRM) enables simultaneous quantification of multiple APIs with high selectivity by targeting specific precursor-to-product ion transitions, minimizing interference from matrix components [54]. Such hyphenated techniques have become essential in the pharmaceutical industry, especially for stability-indicating assays, pharmacokinetic profiling, and bioequivalence studies, where detailed identification and quantification of multiple components are critical [55].

3.4 Sample Preparation

Sample preparation plays a pivotal role in enhancing analyte recovery, mitigating matrix interferences, and ensuring analytical reproducibility in HPLC-based analysis of combination drug products. Depending on the complexity of the sample and analytical requirements, techniques may range from simple dilution or membrane filtration to more advanced and selective procedures such as protein precipitation, liquid-liquid extraction (LLE), and solid-phase extraction (SPE) [56]. In high-

throughput analytical environments, automated sample preparation platforms are increasingly utilized to streamline workflows, minimize operator variability, and enhance processing efficiency [57]. The selection of an appropriate preparation method is contingent upon the physicochemical properties of the analytes, sample matrix complexity, and the sensitivity required for quantification. To align with green analytical chemistry principles and improve operational efficiency, emerging microextraction techniques—including solid-phase microextraction (SPME), dispersive liquid-liquid microextraction (DLLME), and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)—are being adopted as sustainable alternatives. These techniques not only reduce solvent consumption and processing time but also offer high extraction efficiency and compatibility with complex matrices [58].

3.5 Method Validation

According to the ICH Q2(R1) guideline, comprehensive method validation encompasses the evaluation of several critical performance characteristics:

- **Specificity/Selectivity:** The method's ability to unequivocally distinguish the analyte(s) from other components such as impurities, degradation products, and matrix constituents.
- **Linearity:** The proportionality and correlation between the analytical response and analyte concentration across the defined range.
- **Accuracy:** The degree of agreement between the measured values and the true or accepted reference values.
- **Precision:** Including both intra-day repeatability and inter-day intermediate



precision, reflecting the method's reproducibility under consistent conditions.

- **Limit of Detection (LOD) and Limit of Quantitation (LOQ):** The smallest amount of analyte that can be reliably detected or quantified with acceptable accuracy and precision.
- **Robustness:** The capacity of the analytical method to remain unaffected by small, deliberate variations in procedural parameters, ensuring reliability during routine use ^[59].

Each analytical method must be validated separately for every active pharmaceutical ingredient (API) and relevant impurities to confirm its suitability for the intended analytical application ^[60].

4. Case Studies of HPLC Methods Developed for Specific Combination Drugs

4.1 Antihypertensive Combinations

A gradient reversed-phase HPLC method was developed employing a C18 column with a phosphate buffer (pH 3.5) and methanol as the mobile phases to simultaneously analyze amlodipine and valsartan in tablet formulations. Baseline separation was achieved within ten minutes. The method exhibited excellent linearity with precision values below 2% relative standard deviation (RSD) across a concentration range of 2–20 µg/mL. Forced degradation studies confirmed the method's capability as stability-indicating. This validated approach has been successfully implemented for routine quality control, fulfilling ICH guidelines ^[26]. Similarly, an optimized method for losartan and hydrochlorothiazide was established using a phenyl-hexyl column under gradient elution with UV detection. To tackle challenges such as matrix

interference and co-elution, a Quality by Design (QbD) approach utilizing Design of Experiments (DoE) was applied, enhancing method robustness and selectivity ^[61].

4.2 Antidiabetic Combinations

reported an HPLC method coupled with photodiode array (PDA) detection for the simultaneous quantification of glimepiride and metformin in human plasma. Utilizing gradient elution on a C18 column combined with solid-phase extraction (SPE) for sample cleanup, the method demonstrated excellent sensitivity and selectivity, rendering it suitable for pharmacokinetic studies. Validation parameters, including recovery, accuracy, and precision, complied with regulatory standards ^[4]. Additionally, other studies have employed LC-MS/MS techniques to analyze antidiabetic drug combinations in biological matrices, benefiting from superior sensitivity and selectivity, particularly for low-dose analytes ^[62].

4.3 Antibiotics Combinations

developed an isocratic RP-HPLC method for the separation of amoxicillin and clavulanic acid in pharmaceutical dosage forms, achieving baseline separation in under 15 minutes. The method was thoroughly validated for accuracy, precision, linearity, and specificity. Its stability-indicating capability was confirmed by analyzing samples subjected to acidic, basic, and oxidative stress conditions ^[9]. An example of recent advancement is the use of UHPLC-MS/MS, which offers high throughput and enhanced sensitivity for the simultaneous quantification of multiple antibiotics and their metabolites in biological fluids ^[63].

5. Emerging Trends and Future Perspectives



5.1 Use of Hyphenated Techniques (LC-MS/MS, UHPLC)

The use of hyphenated techniques such as LC-MS/MS and UHPLC has grown significantly in response to the increasing complexity of biological matrices and combination drug products. Compared to conventional HPLC, these advanced techniques provide higher sensitivity, enhanced resolution, and significantly reduced run times^[64]. UHPLC, with its sub-2 μm particle columns, achieves superior chromatographic efficiency, allowing complex mixtures to be separated within minutes^[65]. LC-MS/MS further advances impurity profiling and metabolite identification by enabling simultaneous quantification and detailed structural elucidation of analytes^[66]. Consequently, these methods have become indispensable tools in bioanalysis and pharmaceutical development.

5.2 Automation and Artificial Intelligence in Method Development

Automation significantly minimizes human error while markedly enhancing throughput in sample preparation, instrument operation, and data processing workflows^[67]. Cutting-edge method development strategies increasingly leverage artificial intelligence (AI) and machine learning (ML) algorithms to systematically optimize chromatographic parameters by harnessing predictive modeling and extensive historical datasets^[68]. AI-driven platforms can discern key variables, accurately forecast retention behaviors, and propose optimized analytical conditions, thereby expediting development cycles and substantially reducing empirical trial-and-error experimentation^[69].

5.3 Green Analytical Chemistry Approaches

Environmental sustainability is gaining prominence within analytical chemistry. Green

analytical methodologies aim to enhance energy efficiency, minimize hazardous waste generation, and reduce solvent consumption^[70]. Compared to conventional HPLC, techniques such as ultra-high-performance liquid chromatography (UHPLC) and supercritical fluid chromatography (SFC) offer significant reductions in solvent usage and waste output^[71]. Sample preparation approaches, including microextraction, further contribute to waste minimization and decreased reagent consumption^[72]. The adoption of green analytical standards aligns pharmaceutical analysis with broader global sustainability objectives.

6. CONCLUSION

The development of HPLC methods for the analysis of combination drug products presents multifaceted challenges due to the necessity of resolving multiple analytes with diverse physicochemical properties within complex sample matrices. Critical issues—including co-elution, matrix interferences, analyte stability, and rigorous regulatory requirements—demand a strategic, systematic, and integrative approach to method development. The application of Quality by Design (QbD) principles, coupled with state-of-the-art chromatographic and detection technologies, optimized sample preparation techniques, and stringent validation protocols, underpins the establishment of robust, reliable, and regulatory-compliant analytical methods. Empirical evidence from case studies across various therapeutic categories underscores practical strategies and solutions for overcoming analytical complexities. Furthermore, emerging advancements such as hyphenated LC-MS/MS platforms, automation technologies, artificial intelligence-driven method optimization, and environmentally sustainable (green) analytical practices are poised to significantly enhance



analytical efficiency and performance. Sustained innovation, alongside rigorous adherence to evolving regulatory standards, remains essential to ensure the consistent quality, safety, and efficacy of combination pharmaceuticals within an increasingly complex and dynamic pharmaceutical environment.

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HOW TO CITE: Saurabh Borkar*, Pallavi Daf, Anup Barsagade, Combination Drug Analysis: Challenges and Approaches in HPLC Method Development, *Int. J. of Pharm. Sci.*, 2025, Vol 3, Issue 6, 1822-1833. <https://doi.org/10.5281/zenodo.15624782>

