"Recent Advances in RP-HPLC Method Development and Validation for Simultaneous Estimation of ETABONATE and Levofloxacin in Combined Dosage Forms"

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Abstract.
This review provides a thorough and updated summary of recent advancements in reversed-phase high performance liquid chromatography (RP-HPLC) methods developed for the **simultaneous estimation of etabonate and levofloxacin** in combined pharmaceutical dosage forms. It covers the principles of method development, optimization of chromatographic conditions, relevant validation parameters according to ICH guidelines, and the analytical performance of selected methods.
This article also underscores the importance of reliable analytical techniques for ensuring quality control of fixed-dose combinations containing both drugs, which are frequently used due to their combined therapeutic effects.

Keywords:
RP-HPLC, Method Development, Validation, Etabonate, Levofloxacin, Simultaneous Estimation, Combined Dosage Form, ICH Guidelines

1. Introduction

Reversed-phase high performance liquid chromatography (RP-HPLC) is a widely used analytical technique for the simultaneous determination of multiple drug substances in complex pharmaceutical formulations [1].
It offers excellent sensitivity, specificity, and robustness, making it suitable for routine quality control analysis.
Etabonate, a prodrug or derivative often used in combination therapy, and levofloxacin, a broad-spectrum fluoroquinolone antibiotic, are frequently formulated together to leverage anti-infection and complementary therapeutic benefits (e.g., anti-inflammatory plus antibacterial effects). Developing an RP-HPLC method capable of accurately quantifying both etabonate and levofloxacin simultaneously in combined dosage forms presents unique challenges due to their differing chemical properties, UV absorbance spectra, and retention behaviors in chromatographic systems[1].

2. Principles of RP-HPLC Method Development

Reversed-phase high performance liquid chromatography (RP-HPLC) is a highly efficient and widely employed analytical technique for the separation and quantitative determination of pharmaceutical compounds in bulk drugs and combined dosage forms.
In RP-HPLC, separation of analytes is primarily governed by their **hydrophobic interactions** with a **non-polar stationary phase**, typically composed of octadecylsilane (C18) bonded silica, and a **relatively polar mobile phase** [2].

The stationary phase provides hydrophobic retention, whereby analytes with higher lipophilicity exhibit stronger interactions and hence longer retention times.

The mobile phase generally consists of an aqueous component (water or buffer) combined with an organic modifier such as **acetonitrile or methanol**, which regulates elution strength and analyte solubility.

The **choice of stationary phase** is crucial, as C18 columns are preferred due to their high surface area, chemical stability, and ability to separate compounds with a wide polarity range, such as etabonate and levofloxacin [2].

Etabonate, being relatively lipophilic, and levofloxacin, which is moderately polar and ionizable, exhibit different chromatographic behaviors, necessitating careful method optimization to ensure adequate separation.

Several chromatographic parameters critically influence method performance, including **mobile phase composition, pH, flow rate, column temperature, and detection wavelength**.
The pH of the mobile phase plays a particularly important role in controlling the ionization state of levofloxacin, which contains both acidic and basic functional groups, thereby directly affecting its retention and peak shape [2]

Similarly, the organic solvent concentration impacts elution strength, resolution, and analysis time.

Optimization of an RP-HPLC method involves a **systematic and stepwise variation** of these parameters to achieve baseline separation between etabonate and levofloxacin with **acceptable retention times, symmetric peak shapes, high theoretical plate counts, and adequate resolution**.
The ultimate goal of method development is to obtain a robust, reproducible, and efficient chromatographic system suitable for routine quality control and regulatory compliance.

3. Mobile Phase Selection and Optimization

The mobile phase is one of the most influential factors in RP-HPLC method development, as it directly affects **resolution, selectivity, peak symmetry, sensitivity, and quantitative accuracy**.

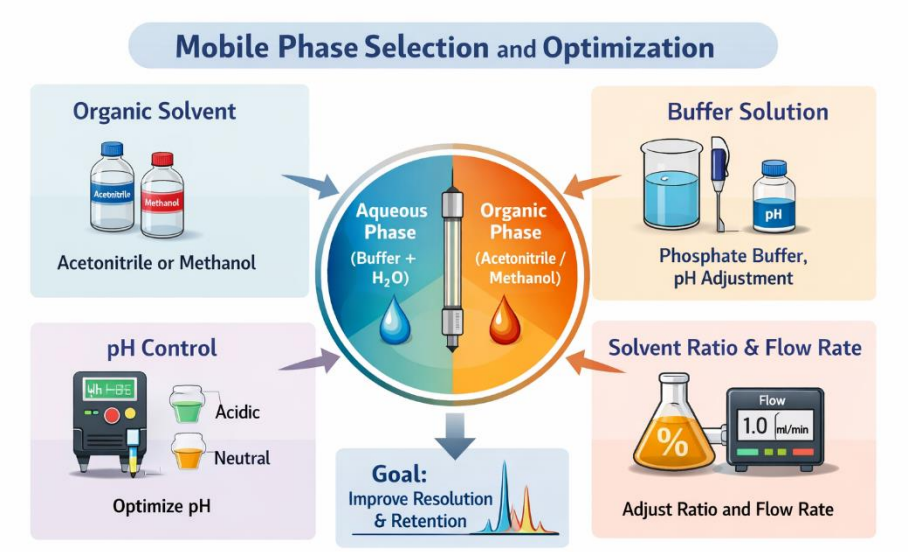
Proper selection and optimization of the mobile phase are essential for achieving reproducible separation of etabonate and levofloxacin in combined dosage forms.

Most RP-HPLC methods utilize a **binary mobile phase system**, consisting of an organic solvent such as **acetonitrile or methanol** and an aqueous component, typically a **buffer solution**. Acetonitrile is often preferred due to its lower viscosity, stronger elution power, and reduced back pressure, while methanol may be selected for its different selectivity and cost-effectiveness.

The aqueous component is commonly a **phosphate buffer**, as it provides good buffering capacity, chemical stability, and compatibility with UV detection.

Adjustment of buffer pH is a critical step, since it influences the ionization state of levofloxacin and thus its retention behavior [3,4].

Maintaining the mobile phase pH below the pKa of levofloxacin ensures controlled ionization, leading to sharper peaks and improved reproducibility.



Literature reports indicate that a mobile phase composed of **acetonitrile and phosphate buffer adjusted to a mildly acidic pH (approximately 3.0–3.5)** provides effective separation of drugs similar to etabonate and levofloxacin in combined pharmaceutical formulations. At this pH range, levofloxacin exhibits stable retention while minimizing peak tailing caused by silanol interactions on the stationary phase, and etabonate elutes with good symmetry and resolution.

The ratio of organic solvent to buffer is optimized to balance **retention time and resolution**, ensuring that both analytes elute within a short analytical run while maintaining baseline separation.

Such optimized mobile phase systems typically result in **retention times of only a few minutes per analyte**, making the method economical, rapid, and suitable for high-throughput routine analysis.

Overall, careful mobile phase selection and optimization play a decisive role in achieving a validated RP-HPLC method with high accuracy, precision, robustness, and regulatory acceptability for simultaneous estimation of etabonate and levofloxacin [5].

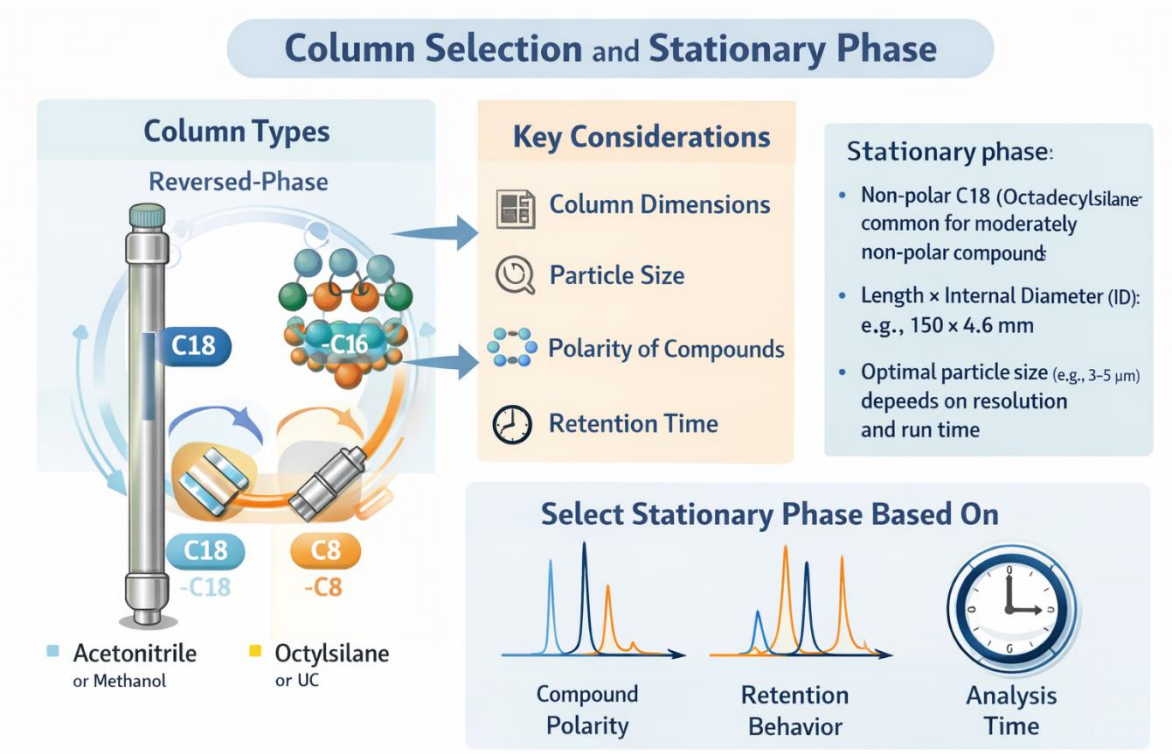
4. Column Selection and Stationary Phase.

A **C18 reversed-phase column** is the most preferred choice due to its non-polar characteristics and ability to retain moderately polar to non-polar compounds.

Column dimensions (length × internal diameter) and particle size (e.g., 4.6 × 150 mm, 5 µm) are selected to balance resolution and analysis time.

Shorter columns can reduce run time but may compromise separation if analytes are similar in polarity.

Other stationary phases (e.g., C8) can be explored for further method refinement if required [6].

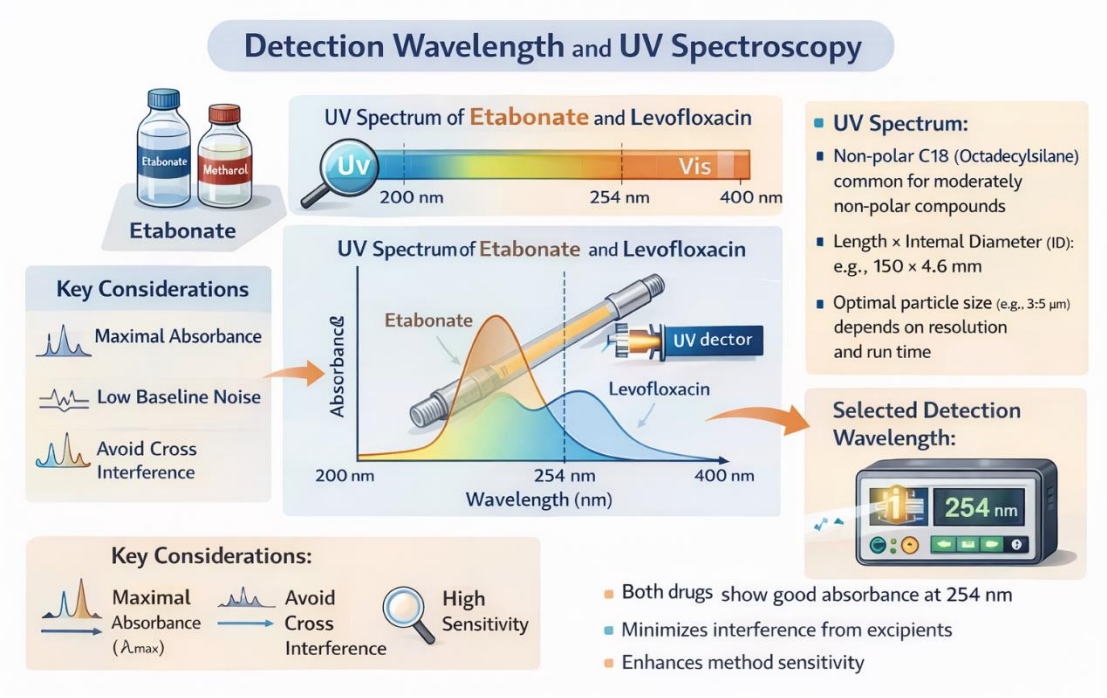


5. Detection Wavelength and UV Spectroscopy

UV detection remains the standard for RP-HPLC due to its sensitivity and simplicity for organic drugs like etabonate and levofloxacin.

Choosing a detection wavelength is based on maximum absorbance (λmax) of both compounds, ensuring that both analytes have adequate response without interference.

Wave lengths around 254–295 nm are commonly used depending on the individual analyte UV profiles and mobile phase composition [7].



6. Method Validation According to ICH Guidelines.

Validation of analytical methods is a critical step in pharmaceutical analysis to ensure that the developed method consistently produces **accurate, precise, reliable, and reproducible results**. For RP-HPLC methods intended for simultaneous estimation of etabonate and levofloxacin in combined dosage forms, validation is performed in accordance with the **International Council for Harmonisation (ICH) guideline Q2(R1)**, which outlines the essential performance characteristics required for analytical procedures [8].

ICH Q2(R1) establishes standardized criteria for evaluating analytical method suitability for quality control, regulatory submission, and routine pharmaceutical analysis. The validation process confirms that the RP-HPLC method is fit for its intended purpose and capable of detecting, identifying, and quantifying both analytes without interference.

The key validation parameters evaluated include **linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), specificity, robustness, and system** suitability [9].

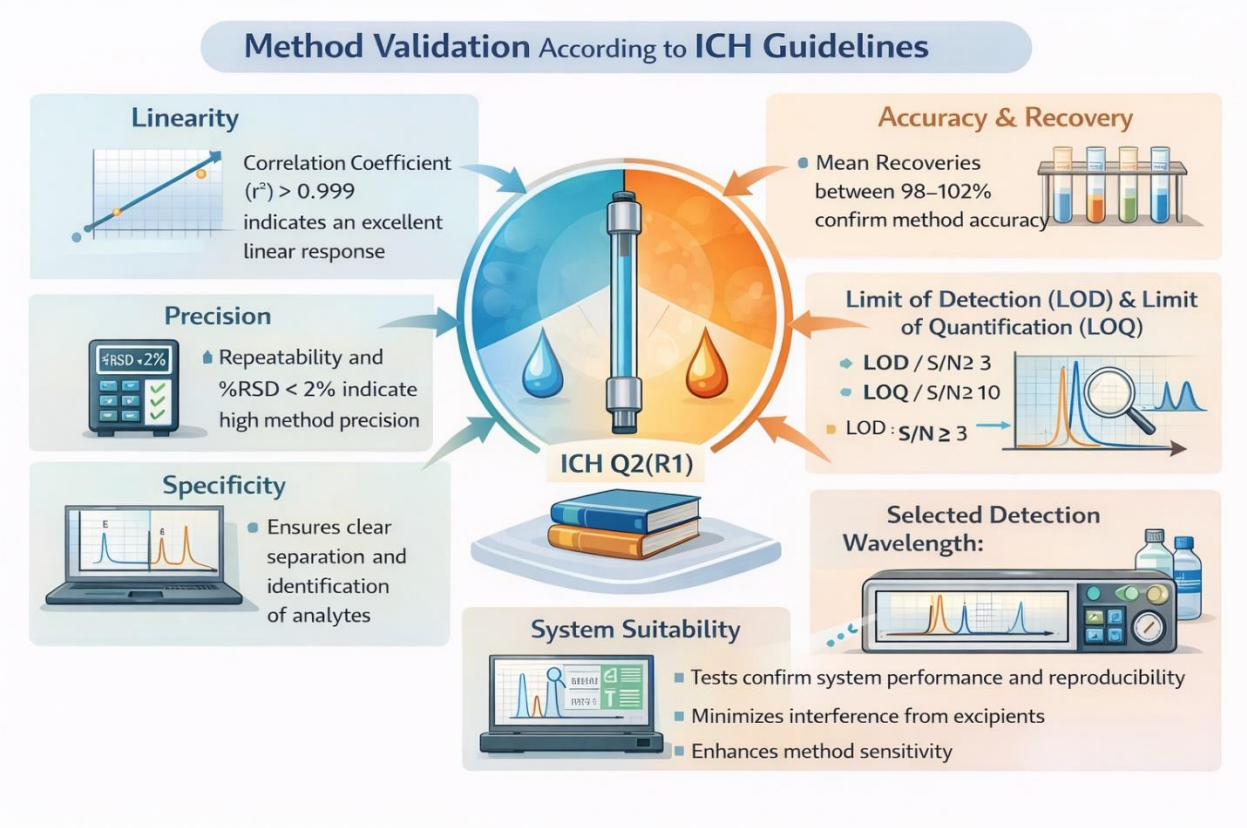
6.1 Linearity

Linearity refers to the ability of the analytical method to elicit test results that are **directly proportional to the concentration of analyte** within a specified range. For RP-HPLC analysis of etabonate and levofloxacin, linearity is typically evaluated by preparing standard solutions at multiple concentration levels covering the expected working range.

Calibration curves are constructed by plotting **peak area versus corresponding analyte concentration**, and linear regression analysis is performed to calculate the correlation coefficient (r^2), slope, and intercept.

A correlation coefficient (r^2) value **greater than 0.999** is commonly reported for well-developed RP-HPLC methods, indicating excellent linear relationships across the tested concentration ranges.

High linearity ensures accurate quantification of both etabonate and levofloxacin at different concentration levels in combined dosage forms and supports the method's applicability for routine assay analysis [10].



6.2 Accuracy and Recovery

Accuracy expresses the **closeness of agreement between the true value and the value obtained using the analytical method**.

In RP-HPLC validation, accuracy is commonly evaluated through **recovery studies** using the standard addition method.

Known quantities of etabonate and levofloxacin standards are added to pre-analyzed sample solutions at different levels, typically **80%, 100%, and 120% of the nominal concentration**. The spiked samples are then analyzed using the developed method, and the percentage recovery of each analyte is calculated [11].

Mean recovery values within the range of **98–102%** indicate that the method accurately quantifies both drugs without interference from excipients or formulation components. Consistent recovery results demonstrate the method's reliability for routine quality control and confirm the absence of systematic analytical errors [12].

6.3 Precision

Precision reflects the degree of **repeatability and reproducibility** of the analytical method under normal operating conditions.
It is evaluated in terms of **intra-day (repeatability)** and **inter-day (intermediate precision)** variations [13].

In intra-day precision studies, multiple replicate injections of the same concentration are analyzed within a single day, whereas inter-day precision assesses variability across different days, analysts, or instruments.

The results are expressed as **percent relative standard deviation (%RSD)** of the peak areas or assay values.

For a validated RP-HPLC method, %RSD values **less than 2%** for both etabonate and levofloxacin are considered acceptable and indicative of high method precision. Low variability confirms that the method produces consistent and reliable results during routine analysis [14].

6.4 Limit of Detection (LOD) and Limit of Quantification (LOQ).

The **limit of detection (LOD)** is defined as the lowest concentration of analyte that can be detected but not necessarily quantified under the stated experimental conditions. The **limit of quantification (LOQ)** represents the lowest concentration that can be quantified with acceptable accuracy and precision [14].

LOD and LOQ are typically determined using **signal-to-noise ratios** of approximately 3:1 and 10:1, respectively, or through statistical calculations based on the **standard deviation of the response and the slope of the calibration curve**.

These sensitivity parameters are crucial for assessing the method's capability to detect and quantify trace levels of etabonate and levofloxacin, particularly in stability studies or low-dose formulations.
Lower LOD and LOQ values indicate higher sensitivity and analytical efficiency of the RP-HPLC method[15].

6.5 Specificity, Robustness, and System Suitability

6.5.1 Specificity

Specificity is the ability of the method to **unequivocally assess the analyte in the presence of components such as excipients, impurities, or degradation products**.
For combined dosage forms, specificity ensures that the peaks corresponding to etabonate and levofloxacin are well resolved and free from interference.
Chromatograms of blank, placebo, standard, and sample solutions are compared to confirm the absence of co-eluting peaks at the retention times of the analytes [16].

6.5.2 Robustness

Robustness evaluates the method's capacity to remain unaffected by **small deliberate variations** in chromatographic conditions, such as minor changes in flow rate, mobile phase composition, pH, or detection wavelength.
A robust RP-HPLC method shows minimal variation in retention time, peak area, and resolution under these modified conditions, demonstrating its reliability during routine use [17].

6.5.3 System Suitability

System suitability tests are performed prior to analysis to verify the adequate performance of the chromatographic system.
Parameters such as **theoretical plate count, tailing factor, resolution, and retention time reproducibility** are assessed to ensure consistent system operation.
Compliance with system suitability criteria confirms that the RP-HPLC system is functioning properly and that the analytical results obtained are valid and reproducible [18].

7. Recent Advances and Case Examples.

In recent years, significant progress has been made in the development of **RP-HPLC methods for simultaneous estimation of combined drug formulations**, particularly those containing levofloxacin in combination with corticosteroids, anti-inflammatory agents, or other antibiotics. These studies highlight improvements in **chromatographic efficiency, analysis time, sensitivity, and method robustness**, thereby enhancing their applicability in routine pharmaceutical quality control.

Advances in column technology, mobile phase optimization, and adherence to ICH validation requirements have enabled researchers to develop **simple, rapid, and reproducible RP-HPLC methods** suitable for fixed-dose combinations with diverse physicochemical properties. Although direct reports on etabonate–levofloxacin combinations are limited, closely related formulations provide valuable methodological insights applicable to this drug pair [19].

7.1 Levofloxacin and Loteprednol Etabonate

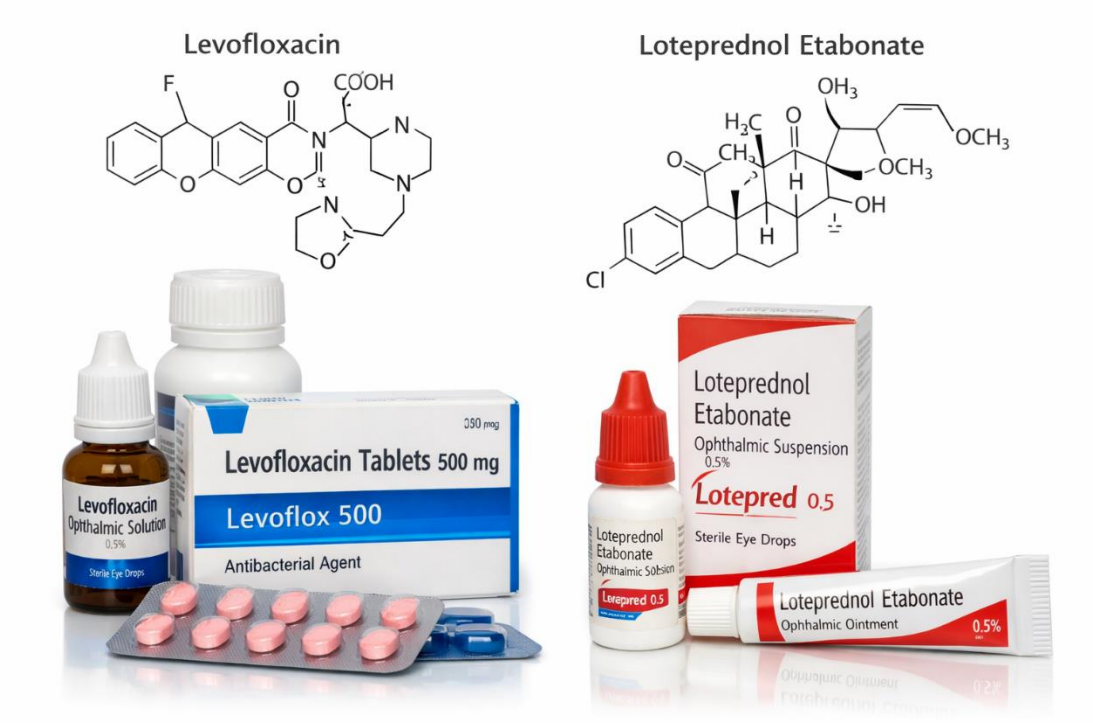
One of the most relevant and well-documented case examples involves the **simultaneous estimation of levofloxacin and loteprednol etabonate** in ophthalmic dosage forms.

This combination is widely used in ophthalmology for the management of bacterial infections accompanied by inflammation, necessitating accurate and reliable analytical methods for quality control.

In reported studies, an RP-HPLC method was successfully developed using a **C18 reversed-phase column**, which provided efficient separation due to its strong hydrophobic interactions and high resolving power [20].

The mobile phase typically consisted of a **phosphate buffer combined with acetonitrile**, optimized to maintain appropriate pH conditions that favor controlled ionization of levofloxacin and stable retention of loteprednol etabonate.

UV detection was performed at **254 nm**, a wavelength selected to ensure adequate absorbance and sensitivity for both analytes without interference from formulation excipients. The optimized chromatographic conditions resulted in **well-resolved, symmetric peaks** with acceptable retention times, allowing rapid analysis suitable for routine laboratory use.



Validation studies conducted in accordance with **ICH Q2(R1) guidelines** demonstrated excellent linearity over the tested concentration ranges, with correlation coefficients exceeding 0.999. Accuracy studies showed recoveries within acceptable limits, while precision results exhibited low %RSD values, confirming the repeatability and reproducibility of the method. Robustness testing further confirmed that minor variations in chromatographic conditions did not

significantly affect method performance, indicating suitability for routine quality control applications [21].

7.2 RP-HPLC in Other Levofloxacin-Based Drug Combinations

Beyond etabonate derivatives, numerous studies have reported the successful development of RP-HPLC methods for the simultaneous estimation of **levofloxacin combined with other antibacterial agents**, such as **azithromycin, cefpodoxime proxetil, and other β-lactam or macrolide antibiotics**.
These drug combinations are commonly used in clinical practice to enhance antimicrobial efficacy and reduce resistance development.

Such studies emphasize the importance of **careful mobile phase optimization**, particularly the selection of organic solvent type and concentration, buffer composition, and pH adjustment. In many cases, acidic phosphate buffers combined with acetonitrile or methanol were found to provide optimal separation, minimize peak tailing, and improve resolution between analytes with differing polarity and ionization characteristics [22].

Detection wavelength selection also played a crucial role, with UV detection commonly employed due to its simplicity and sensitivity.

Optimized detection parameters ensured sufficient response for levofloxacin and its co-formulated drug, even when their UV absorption maxima differed.

The resulting methods consistently demonstrated **high resolution, short run times, and strong validation performance**, including excellent linearity, accuracy, precision, and robustness. These findings underscore the adaptability of RP-HPLC techniques for analyzing complex combined dosage forms [22].

7.3 Relevance to Etabonate–Levofloxacin Method Development

Although published studies specifically addressing the simultaneous estimation of **etabonate and levofloxacin** (beyond loteprednol esters) are limited, the methodologies described in related drug combinations provide **strong scientific and practical guidance**.
The successful strategies employed—such as selection of C18 columns, use of buffered mobile phases at acidic pH, and systematic method validation—can be directly adapted to the development of RP-HPLC methods for etabonate–levofloxacin formulations [22].

These case examples demonstrate that with appropriate optimization of chromatographic parameters, it is feasible to achieve **accurate, precise, and robust simultaneous estimation** of drugs with differing chemical characteristics.

Consequently, the insights gained from recent advances in levofloxacin-based combination analyses form a strong foundation for future analytical method development targeting etabonate and levofloxacin in combined dosage forms.

8. Challenges in Method Development

The simultaneous estimation of two chemically and pharmacologically distinct drugs such as **etabonate and levofloxacin** presents several analytical challenges during RP-HPLC method development.

These challenges primarily arise due to **differences in physicochemical properties**, including polarity, solubility, pKa values, lipophilicity, and UV absorption characteristics.

One of the most common difficulties encountered is **overlapping or closely eluting peaks**, which can compromise resolution and accurate quantification.

Levofloxacin, being moderately polar and ionizable, often exhibits strong interactions with the stationary phase and silanol groups, while etabonate, which is relatively lipophilic, tends to elute later under similar chromatographic conditions.

This disparity in retention behavior can lead to either excessive retention time for one analyte or insufficient separation for the other [23].

Another significant challenge is the **difference in UV absorbance profiles** of the two drugs. Selecting a single detection wavelength that provides adequate sensitivity for both analytes without interference from excipients or degradation products requires careful spectral analysis and optimization.

To overcome these issues, **systematic adjustment of chromatographic parameters** is essential. The pH of the mobile phase must be carefully controlled to regulate the ionization state of levofloxacin, thereby improving peak symmetry and reproducibility. Similarly, buffer strength and composition influence peak shape and system stability, while the organic solvent ratio determines elution strength and overall run time.

Flow rate optimization is also critical, as higher flow rates may reduce analysis time but can negatively impact resolution, whereas lower flow rates may improve separation at the expense of longer run times.

Achieving an optimal balance between **retention, resolution, sensitivity, and analytical efficiency** is therefore a key objective of method development.

In situations where **isocratic elution** fails to provide satisfactory separation due to large differences in analyte retention, **gradient elution techniques** may be employed.

Gradient methods allow gradual changes in mobile phase composition, enhancing separation of compounds with widely differing polarities while maintaining reasonable analysis times. However, gradient methods require additional optimization and system equilibration considerations [24].

9. Applications in Quality Control and Regulatory Compliance

Validated RP-HPLC methods play a **central role in pharmaceutical quality control**, particularly for the analysis of **fixed-dose combination products** containing etabonate and levofloxacin. These methods are routinely employed to ensure **assay determination, content uniformity, dissolution testing, and stability assessment**, thereby confirming product quality, safety, and efficacy.

In routine quality control laboratories, RP-HPLC methods provide **high precision and accuracy**, enabling reliable batch-to-batch consistency checks during manufacturing. They are also essential for detecting deviations in drug content that may arise due to formulation variability, process changes, or storage conditions.

Stability-indicating RP-HPLC methods are especially valuable, as they can distinguish the active pharmaceutical ingredients from degradation products formed under stress conditions such as heat, light, moisture, or oxidative environments. This capability is critical for establishing shelf life and appropriate storage conditions for combined dosage forms [24,25].

From a regulatory perspective, global agencies such as the **U.S. Food and Drug Administration (FDA)**, the **European Medicines Agency (EMA)**, and the **Central Drugs Standard Control Organization (CDSCO)** mandate the use of **validated analytical procedures** in accordance with ICH guidelines for drug approval and post-marketing surveillance. Analytical methods submitted in regulatory dossiers must demonstrate compliance with validation requirements to ensure reliability and reproducibility across different laboratories [26].

Furthermore, validated RP-HPLC methods support **post-marketing quality monitoring**, helping to identify substandard or counterfeit products and ensuring continued compliance with regulatory standards throughout the product lifecycle [27].

10. Conclusion

Reversed-phase high performance liquid chromatography (RP-HPLC) continues to be an indispensable and widely accepted analytical technique for the **simultaneous estimation of active pharmaceutical ingredients in combined dosage forms**.

Its inherent advantages, including **high precision, excellent accuracy, superior resolution, and robust performance**, make RP-HPLC particularly suitable for the analysis of complex fixed-dose combinations such as etabonate and levofloxacin [28].

Recent advancements in chromatographic science have significantly enhanced the reliability and efficiency of RP-HPLC methods.

These advancements include the development of **optimized mobile phase systems**, improved understanding of pH-dependent analyte behavior, and the availability of **advanced column technologies** with better surface chemistry and particle uniformity. Additionally, the adoption of **rigorous method validation strategies in accordance with ICH Q2(R1) guidelines** has strengthened confidence in the analytical performance and regulatory acceptability of these methods.

The development and validation of an RP-HPLC method for the simultaneous estimation of **etabonate and levofloxacin** provide a powerful and reliable analytical tool for pharmaceutical analysis [29].

Such validated methods play a critical role in **routine quality control, stability testing, and regulatory submissions**, ensuring consistency, safety, and therapeutic efficacy of combined dosage forms throughout their product lifecycle.

In conclusion, continued refinement of RP-HPLC methodologies, supported by systematic optimization and comprehensive validation, will remain essential for meeting the evolving analytical and regulatory demands of modern pharmaceutical development and manufacturing [30].

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