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Development and Validation of a Stability-Indicating HPLC Method for the Quantitative Estimation of Enrofloxacin in Pharmaceutical Dosage Forms Under Various Stress Conditions

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ABSTRACT

Enrofloxacin, a widely used fluoroquinolone antibiotic in veterinary medicine, requires stringent quality control to ensure its safety, efficacy, and stability. The development of stability-indicating analytical methods is essential for accurately quantifying the drug in the presence of its degradation products formed under various stress conditions. High-performance liquid chromatography (HPLC), particularly reverse-phase HPLC, has emerged as a reliable and sensitive technique for this purpose.

This review focuses on the development and validation of stability-indicating HPLC methods for the quantitative estimation of enrofloxacin in pharmaceutical dosage forms. Key aspects of method development, including selection of chromatographic conditions, optimization of mobile phase, and detection parameters, are discussed in detail. The role of forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic conditions in establishing method specificity is also highlighted.

Furthermore, the review summarizes validation parameters such as specificity, linearity, accuracy, precision, robustness, and sensitivity in accordance with International Council for Harmonisation (ICH) guidelines. Recent advancements in analytical techniques, including green chromatography and hyphenated methods, are also considered.

Overall, stability-indicating HPLC methods play a crucial role in pharmaceutical quality assurance, regulatory compliance, and stability assessment of enrofloxacin formulations, ensuring the delivery of safe and effective veterinary products.

1. INTRODUCTION

Enrofloxacin is a synthetic fluoroquinolone antibiotic widely used in veterinary medicine for the treatment of a broad range of bacterial infections in livestock and companion animals. It exhibits potent activity against both Gram-positive and Gram-negative microorganisms by inhibiting bacterial DNA gyrase and topoisomerase IV, enzymes essential for DNA replication and transcription [1]. Due to its broad-spectrum antimicrobial activity and favorable pharmacokinetic profile, enrofloxacin is commonly formulated into various pharmaceutical dosage forms such as tablets, injectable solutions, and oral suspensions [2].

The quality, safety, and efficacy of pharmaceutical products are highly dependent on their stability throughout the shelf life. Drug degradation may occur due to exposure to environmental factors such as temperature, light, humidity, pH, and oxidative conditions, leading to the formation of degradation products that may be less effective or potentially toxic [3]. Therefore, it is essential to evaluate the stability

characteristics of drug substances and drug products under various stress conditions. Stability testing plays a crucial role in determining appropriate storage conditions, retest periods, and shelf life of pharmaceutical formulations [4].

In this context, analytical methods used for the estimation of active pharmaceutical ingredients must be capable of accurately quantifying the drug in the presence of its degradation products, impurities, and excipients. Such methods are referred to as stability-indicating methods. According to the International Council for Harmonisation (ICH) guidelines, a stability-indicating method should be able to specifically measure the active drug substance without interference from degradation products, process impurities, or other components present in the formulation [5]. Forced degradation studies, including acid, base, oxidation, thermal, and photolytic stress testing, are commonly employed to demonstrate the specificity and stability-indicating capability of the method [6].

Among various analytical techniques, High-Performance Liquid Chromatography (HPLC) has emerged as one of the most widely used and reliable methods for pharmaceutical analysis. HPLC offers several advantages, including high sensitivity, excellent resolution, reproducibility, and the ability to analyze complex mixtures [7]. Reverse-phase HPLC (RP-HPLC), in particular, is extensively utilized for the analysis of fluoroquinolone antibiotics due to its efficiency in separating compounds of varying polarity [8]. The technique allows precise quantification of enrofloxacin even in the presence of its degradation products, making it an indispensable tool in quality control and stability studies.

In recent years, significant advancements have been made in the development of stability-indicating HPLC methods, with a focus on improving method sensitivity, reducing analysis time, and adopting environmentally friendly (green) analytical approaches. These developments have further enhanced the applicability of HPLC in pharmaceutical research, quality assurance, and regulatory compliance [9].

2. ENROFLOXACIN: DRUG PROFILE

Enrofloxacin is a second-generation fluoroquinolone antibiotic extensively used in veterinary medicine for the treatment of various bacterial infections in animals. It is a synthetic derivative of nalidixic acid and exhibits potent bactericidal activity against a wide range of Gram-positive and Gram-negative organisms. The drug is particularly effective against pathogens such as *Escherichia coli*, *Salmonella spp.*, *Pasteurella spp.*, and *Staphylococcus aureus*, which are commonly associated with respiratory, gastrointestinal, and urinary tract infections in animals [10].

Chemically, enrofloxacin is described as 1-cyclopropyl-6-fluoro-7-(4-ethyl-1-piperazinyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. It belongs to the fluoroquinolone class due to the presence of a fluorine atom at the sixth position of the quinolone ring, which enhances its antibacterial activity and tissue penetration [11]. The molecular formula of enrofloxacin is $C_{19}H_{22}FN_3O_3$, and it has a molecular weight of approximately 359.4 g/mol. It appears as a pale yellow crystalline powder that is slightly soluble in water but more soluble in acidic or alkaline conditions [12].

The mechanism of action of enrofloxacin involves inhibition of bacterial DNA gyrase (topoisomerase II) and topoisomerase IV, which are essential enzymes responsible for DNA replication, transcription, and repair. By interfering with these enzymes, enrofloxacin prevents bacterial cell division, ultimately leading to cell death. This dual mechanism contributes to its broad-spectrum activity and reduced likelihood of resistance development compared to older antibiotics [13].

Pharmacokinetically, enrofloxacin is well absorbed following oral and parenteral administration in animals. It exhibits high bioavailability and extensive tissue distribution, achieving therapeutic concentrations in various organs such as lungs, liver, kidneys, and urinary tract. Enrofloxacin is partially metabolized in the liver to its active metabolite, ciprofloxacin, which further contributes to its antibacterial effect. The drug is primarily excreted through urine and bile, making it effective in treating systemic as well as localized infections [14].

Enrofloxacin is available in multiple pharmaceutical dosage forms, including tablets, injectable solutions, oral suspensions, and medicated feed formulations. These dosage forms are designed to ensure ease of administration and optimal therapeutic efficacy in different animal species. However, the stability of enrofloxacin in these formulations may be affected by environmental factors such as pH, temperature, light, and oxidative conditions, leading to degradation and reduced potency [15].

The stability profile of enrofloxacin indicates that it is susceptible to degradation under acidic and alkaline conditions, as well as upon exposure to light and oxidizing agents. Degradation products formed under such stress conditions may compromise drug safety and efficacy. Therefore, it is essential to develop and validate stability-indicating analytical methods capable of accurately detecting and quantifying enrofloxacin in the presence of its degradation products [16].

In addition, regulatory authorities emphasize strict quality control of veterinary pharmaceuticals to ensure food safety and prevent the development of antimicrobial resistance. Residues of enrofloxacin in animal-derived food products such as milk, meat, and eggs are a major concern, necessitating the use of sensitive and specific analytical methods for monitoring and control [17].

3. NEED FOR STABILITY-INDICATING METHODS

Stability-indicating analytical methods play a crucial role in pharmaceutical analysis by ensuring that the active pharmaceutical ingredient (API) can be accurately quantified in the presence of its degradation products, impurities, and excipients. These methods are specifically designed to provide reliable information about the stability characteristics of a drug substance and its formulations under various environmental conditions [18].

One of the primary objectives of stability-indicating methods is to determine the intrinsic stability of the drug. Intrinsic stability refers to the inherent susceptibility of a drug molecule to undergo chemical degradation when exposed to stress factors such as heat, light, pH changes, and oxidation. Understanding the intrinsic stability helps in predicting degradation behavior and selecting appropriate formulation strategies and storage conditions [19].

Another important aspect is the identification of degradation pathways. During stability studies, drugs may undergo hydrolysis, oxidation, photolysis, or thermal degradation, leading to the formation of multiple degradation products. Stability-indicating methods enable the separation and detection of these products, thereby helping to elucidate the degradation mechanisms and pathways of the drug [20].

Ensuring product quality throughout its shelf life is also a key requirement in pharmaceutical development. Degradation of the drug substance can result in loss of potency and formation of toxic or inactive compounds, which may compromise patient safety. Stability-indicating methods provide accurate and reproducible results that support stability testing, shelf-life determination, and quality control of pharmaceutical products [21].

Furthermore, these methods are essential for meeting regulatory requirements. Regulatory authorities such as the International Council for Harmonisation (ICH), USFDA, and EMA mandate the use of validated stability-indicating methods for the analysis of drug substances and products. According to ICH guidelines, analytical methods must demonstrate specificity, which is the ability to assess the analyte unequivocally in the presence of expected components such as impurities, degradation products, and matrix components [22].

To establish the stability-indicating nature of an analytical method, forced degradation studies are recommended. These studies involve subjecting the drug to extreme conditions such as acidic and alkaline hydrolysis, oxidation, thermal stress, and photolysis. The purpose is to generate degradation products and demonstrate that the analytical method can effectively separate and quantify the drug without interference. Such studies provide valuable information regarding degradation kinetics and help in validating the robustness and specificity of the method [23].

4. PRINCIPLE OF HPLC IN DRUG ANALYSIS

High-Performance Liquid Chromatography (HPLC) is a powerful analytical technique widely used for the separation, identification, and quantification of components present in pharmaceutical formulations. The principle of HPLC is based on the differential distribution of analytes between a stationary phase and a mobile phase, resulting in separation due to differences in their physicochemical properties such as polarity, molecular size, and interaction with the stationary phase [24].

In HPLC, the sample mixture is introduced into a column packed with a stationary phase, while a liquid mobile phase is pumped through the column at high pressure. As the analytes travel through the column, they interact differently with the stationary phase, leading to separation. Compounds with stronger interactions with the stationary phase elute later, while those with weaker interactions elute earlier, resulting in distinct retention times [25].

For the analysis of enrofloxacin, reverse-phase HPLC (RP-HPLC) is most commonly employed. In RP-HPLC, the stationary phase is non-polar (typically C18 silica), and the mobile phase is relatively polar, usually consisting of a mixture of water or buffer and an organic solvent such as methanol or acetonitrile. This setup is particularly suitable for moderately polar compounds like enrofloxacin, allowing efficient separation from its degradation products and impurities [26].

Detection in HPLC is commonly carried out using a UV-visible detector, as enrofloxacin exhibits strong absorbance in the UV region. The detection wavelength is typically selected in the range of 270–280 nm, corresponding to the maximum absorbance (λmax) of the drug, ensuring high sensitivity and accurate quantification [27].

Quantitative analysis in HPLC is based on parameters such as peak area, retention time, and resolution. The peak area is directly proportional to the concentration of the analyte, allowing precise quantification using calibration curves. Retention time helps in identifying the compound, while resolution ensures adequate separation between the drug and its degradation products or impurities [28].

Overall, HPLC offers several advantages, including high sensitivity, specificity, reproducibility, and the ability to analyze complex mixtures. These characteristics make it an indispensable tool for the development of stability-indicating methods in pharmaceutical analysis [29].

5. METHOD DEVELOPMENT

Method development is a critical step in establishing a reliable and reproducible analytical procedure for the quantitative estimation of enrofloxacin. It involves systematic optimization of chromatographic parameters to achieve efficient separation of the drug from its impurities and degradation products. The primary objective is to obtain a method that is accurate, precise, specific, and robust, while providing well-resolved and symmetrical peaks within a reasonable analysis time [30].

5.1 Selection of Chromatographic Conditions

The selection of appropriate chromatographic conditions is essential for achieving optimal separation and accurate quantification of enrofloxacin. Various parameters must be carefully optimized during method development.

The choice of column plays a significant role in separation efficiency. Reverse-phase columns, particularly C18 (octadecylsilane) columns, are most commonly used due to their non-polar nature and compatibility with a wide range of compounds. These columns provide excellent retention and resolution for moderately polar compounds such as enrofloxacin [31].

The mobile phase composition is another critical factor influencing separation. It typically consists of a combination of an aqueous buffer and an organic solvent such as methanol or acetonitrile. The ratio of these components is optimized to achieve adequate retention time, good peak shape, and effective separation from impurities and degradation products [32].

The flow rate of the mobile phase is optimized to balance analysis time and resolution. Higher flow rates may reduce analysis time but can compromise resolution, whereas lower flow rates improve separation but increase run time. Therefore, an optimal flow rate is selected to ensure efficient chromatographic performance [33].

The detection wavelength is selected based on the UV absorption characteristics of enrofloxacin. Choosing an appropriate wavelength ensures maximum sensitivity and accurate detection of the analyte [34].

The injection volume is also optimized to ensure reproducibility and prevent column overloading. Excessive injection volume may lead to peak broadening and poor resolution, while too small a volume may reduce sensitivity [35].

Overall, the aim of selecting chromatographic conditions is to achieve sharp and symmetrical peaks, good resolution between analyte and impurities, minimal peak tailing, and reproducible retention times, which are essential for a reliable analytical method [36].

5.2 Optimization of Mobile Phase

Optimization of the mobile phase is a key aspect of method development, as it directly affects the separation, retention, and peak shape of analytes. The mobile phase must be carefully selected to ensure proper interaction between the analyte, stationary phase, and solvent system [37].

For enrofloxacin analysis, the mobile phase generally consists of a mixture of an aqueous buffer and an organic solvent such as acetonitrile or methanol. The pH of the mobile phase is particularly important because enrofloxacin is an amphoteric compound, and its ionization state can significantly influence its retention behavior. Maintaining an appropriate pH ensures consistent retention time and improved peak symmetry [38].

Buffers such as phosphate buffer are commonly used due to their ability to maintain stable pH and compatibility with HPLC systems. The buffer concentration is also optimized to provide adequate buffering capacity without causing excessive backpressure or interference with detection [39].

The proportion of organic solvent is adjusted to achieve a suitable balance between retention time and resolution. Increasing the organic solvent content generally decreases retention time, whereas decreasing it increases retention and improves separation [40].

Proper optimization of the mobile phase results in clear separation of enrofloxacin from its impurities and degradation products, symmetrical peak shape, and acceptable retention time, which are critical for accurate quantitative analysis.

5.3 Detection Wavelength Selection

Selection of an appropriate detection wavelength is essential for achieving high sensitivity and specificity in HPLC analysis. Enrofloxacin exhibits strong absorption in the ultraviolet (UV) region due to its quinolone structure [41].

The detection wavelength is typically selected based on the maximum absorbance (λmax) of the drug, which lies in the range of 270–280 nm. At this wavelength, the drug exhibits maximum absorbance, resulting in enhanced sensitivity and improved signal-to-noise ratio [42].

Choosing an appropriate wavelength also minimizes interference from excipients, impurities, and degradation products, thereby improving the selectivity of the method. In some cases, a wavelength scan is performed using a UV spectrophotometer to determine the exact λmax before finalizing the detection wavelength [43].

Thus, proper selection of the detection wavelength ensures accurate, sensitive, and reproducible quantification of enrofloxacin in pharmaceutical dosage forms.

6. FORCED DEGRADATION STUDIES

Forced degradation studies are an integral part of analytical method development, particularly for establishing the stability-indicating nature of an HPLC method. These studies involve subjecting the drug substance and drug product to extreme stress conditions to accelerate degradation and generate potential degradation products. The primary objective is to demonstrate that the analytical method can effectively separate and quantify the active pharmaceutical ingredient (API) in the presence of its degradation products, impurities, and excipients [44].

Forced degradation also provides valuable information about the intrinsic stability of the drug molecule, helps in identifying degradation pathways, and supports the development of stable formulations. According to regulatory guidelines, stress testing should include hydrolytic (acidic and alkaline), oxidative, thermal, and photolytic conditions to ensure comprehensive evaluation of drug stability [45].

6.1 Acidic Degradation

Acidic degradation is performed by treating the drug with a strong acid, typically hydrochloric acid (HCl), under controlled conditions. This study evaluates the susceptibility of the drug to hydrolysis in acidic environments. Enrofloxacin may undergo structural changes under acidic conditions, leading to the formation of degradation products that must be separated and detected by the analytical method [46].

6.2 Alkaline Degradation

Alkaline degradation is carried out using a strong base such as sodium hydroxide (NaOH). Basic conditions can induce hydrolytic degradation, particularly in compounds containing functional groups sensitive to alkaline pH. Evaluating degradation under such conditions helps in understanding the stability profile of enrofloxacin in alkaline environments [47].

6.3 Oxidative Degradation

Oxidative degradation studies are performed using oxidizing agents such as hydrogen peroxide (H_2O_2). These conditions simulate oxidative stress that may occur during manufacturing, storage, or handling of the drug product. Enrofloxacin may undergo oxidation leading to the formation of oxidative degradation products, which must be effectively resolved during chromatographic analysis [48].

6.4 Thermal Degradation

Thermal degradation involves exposing the drug to elevated temperatures, typically in the range of 60–80°C, for a specified period. This study assesses the thermal stability of the drug and identifies degradation products formed due to heat exposure. Thermal stress testing is important for determining appropriate storage conditions and packaging requirements [49].

6.5 Photolytic Degradation

Photolytic degradation is conducted by exposing the drug to ultraviolet (UV) light or direct sunlight. Light exposure can cause photochemical reactions leading to drug degradation. This study helps evaluate the photosensitivity of enrofloxacin and ensures that the analytical method can detect and separate photodegradation products [50].

7. METHOD VALIDATION

Method validation is a systematic process used to confirm that an analytical method is suitable for its intended purpose. Validation of HPLC methods is performed according to guidelines such as ICH Q2(R1)**,** which specify various parameters to ensure the reliability, accuracy, and reproducibility of the analytical procedure [51].

7.1 Specificity

Specificity is the ability of the analytical method to unequivocally assess the analyte in the presence of other components such as impurities, degradation products, and excipients. A stability-indicating method must demonstrate that enrofloxacin can be accurately measured without interference from these components [52].

7.2 Linearity

Linearity refers to the ability of the method to produce results that are directly proportional to the concentration of the analyte within a specified range. It is typically evaluated by preparing calibration standards at different concentration levels and plotting a calibration curve of peak area versus concentration [53].

7.3 Accuracy

Accuracy indicates the closeness of the measured value to the true value. It is commonly assessed by recovery studies, where known amounts of enrofloxacin are added to the sample matrix and analyzed. The percentage recovery should fall within acceptable limits, usually 98–102% [54].

7.4 Precision

Precision measures the degree of reproducibility of the analytical method under normal operating conditions. It is evaluated at two levels:

- **Repeatability (intra-day precision)**
- **Intermediate precision (inter-day or different analyst/instrument)**

Results are expressed as percentage relative standard deviation (%RSD), which should be within acceptable limits [55].

7.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD is the lowest concentration of the analyte that can be detected but not necessarily quantified, whereas LOQ is the lowest concentration that can be quantitatively determined with acceptable accuracy and precision. These parameters indicate the **sensitivity** of the analytical method [56].

7.6 Robustness

Robustness evaluates the reliability of the analytical method under small, deliberate variations in method parameters such as flow rate, mobile phase composition, pH, and temperature. A robust method remains unaffected by such variations, indicating its suitability for routine use [57].

7.7 System Suitability Testing

System suitability testing is performed before sample analysis to ensure that the HPLC system is functioning properly. Parameters such as retention time, theoretical plates, tailing factor, and resolution are evaluated to confirm system performance and reliability [58].

8. APPLICATIONS OF THE DEVELOPED METHOD

The development of a validated stability-indicating HPLC method for enrofloxacin has wide-ranging applications in pharmaceutical analysis and quality assurance. Such methods are essential for ensuring the safety, efficacy, and regulatory compliance of drug products throughout their lifecycle [59].

One of the primary applications is in routine quality control of enrofloxacin formulations. The method can be used for the accurate estimation of the drug content in various dosage forms such as tablets, injections,

and oral suspensions. Its high specificity ensures that the active ingredient is quantified without interference from excipients or impurities [60].

Another important application is stability testing during product development. Stability-indicating methods are used to monitor the degradation behavior of enrofloxacin under different environmental conditions such as temperature, humidity, and light. This helps in determining shelf life, storage conditions, and packaging requirements for pharmaceutical products [61].

The method is also valuable for the detection and quantification of degradation products formed during forced degradation studies or long-term stability studies. Identification and separation of these degradation products are critical for assessing the safety and quality of the drug product [62].

Furthermore, such validated methods are essential for regulatory submissions. Regulatory authorities require comprehensive analytical data demonstrating the specificity, accuracy, precision, and robustness of the method. Stability-indicating HPLC methods support documentation for drug approval processes and ensure compliance with international regulatory guidelines [63].

9. CHALLENGES IN METHOD DEVELOPMENT

Despite the advantages of HPLC, the development of a robust stability-indicating method for enrofloxacin presents several challenges that must be carefully addressed.

One of the major challenges is the separation of closely related degradation products. Enrofloxacin may undergo multiple degradation pathways, producing structurally similar compounds that are difficult to resolve chromatographically. Achieving adequate resolution between these components requires careful optimization of chromatographic conditions [64].

The selection of an appropriate mobile phase is another critical challenge. The composition, pH, and proportion of organic solvent significantly influence retention time, peak shape, and resolution. Improper selection may lead to poor separation, peak tailing, or co-elution of analytes and impurities [65].

Maintaining reproducibility and robustness of the method is also essential. Variations in experimental conditions such as flow rate, temperature, and column performance can affect the reliability of the results. Therefore, the method must be optimized and validated to ensure consistent performance under routine laboratory conditions [66].

Another important challenge is achieving sufficient sensitivity for detecting low-level impurities and degradation products. Analytical methods must be capable of detecting trace levels of these components to meet regulatory requirements and ensure product safety [67].

10. FUTURE PERSPECTIVES

Advancements in analytical technologies are continuously improving the efficiency and reliability of pharmaceutical analysis. Techniques such as liquid chromatography–mass spectrometry (LC–MS) and ultra-performance liquid chromatography (UPLC) offer enhanced sensitivity, selectivity, and faster analysis compared to conventional HPLC methods. These techniques are increasingly being used for the identification and quantification of trace-level impurities and degradation products [68].

In addition, there is a growing emphasis on the development of eco-friendly or green analytical methods. Green chromatography focuses on reducing the use of hazardous solvents, minimizing waste generation, and improving energy efficiency without compromising analytical performance. The adoption of such sustainable practices is becoming an important consideration in modern pharmaceutical analysis [69].

Future research is also directed toward automation, miniaturization, and the use of advanced data analysis tools, which can further improve method efficiency and reproducibility. These developments will enhance the applicability of stability-indicating methods and support the evolving needs of pharmaceutical quality assurance and regulatory compliance [70].

11. CONCLUSION

The development and validation of a stability-indicating HPLC method for the quantitative estimation of enrofloxacin are essential for ensuring the quality, safety, and efficacy of pharmaceutical dosage forms. Such methods enable accurate determination of the drug in the presence of its degradation products, impurities, and excipients, thereby fulfilling critical requirements for pharmaceutical analysis and regulatory compliance.

Forced degradation studies play a vital role in establishing the stability-indicating capability of the method by revealing the intrinsic stability of enrofloxacin and identifying its degradation pathways under various stress conditions, including acidic, alkaline, oxidative, thermal, and photolytic environments. These studies confirm the specificity and reliability of the analytical method.

Method validation, performed in accordance with internationally accepted guidelines, ensures that the developed HPLC method is accurate, precise, sensitive, robust, and reproducible. The optimized chromatographic conditions, including selection of column, mobile phase composition, and detection wavelength, contribute to effective separation and quantification of enrofloxacin.

Overall, stability-indicating HPLC methods remain indispensable tools in routine quality control, stability testing, and regulatory submissions. With ongoing advancements in analytical technologies and increasing emphasis on green chemistry, future developments are expected to further enhance the efficiency, sensitivity, and sustainability of such methods in pharmaceutical analysis.

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