# Study of Stability Profiles and Development of New Stability Indicating Analytical Methods for Selected Fluoroquinolones

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By

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## DECLARATION

I, Rupali Keny (Sachi Kudchadkar) hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any otherUniversity or Institution for the award of any research degree.

Place: Taleigao Plateau.

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## CERTIFICATE

I hereby certify that the work was carried out under my supervision and may be placed for evaluation.

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# Acknowledgments

"Education is a lifelong journey whose destination expands as you travel."
— Jim Stovall

And it is said the journey is more beautiful than the destination.

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# DEDICATED TO

# THE LIGHT OF MY LIFE

# MY SON

# ISHAAN

*I want you to believe, deep in your heart…*
*That you are capable of achieving anything you put your mind to…*

*That you will never lose, you either win or you learn…*
*Just go ahead… learn …*
*and win hearts along the way…*

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# List of Abbreviations

| **Abbreviation……** | **Full Form** |
|---|---|
| % …… | Percentage |
| % RSD…… | Percentage Relative Standard Deviation |
| °C…… | Degree Centigrade |
| ACN…… | Acetonitrile |
| API…… | Active Pharmaceutical Ingredient |
| AR…… | Analytical Reagent |
| BP……. | British Pharmacopoeia |
| CDA……. | Acid hydrolytic Degradant of CIP |
| CDO……. | Oxidative Degradant of CIP |
| CIP…… | Ciprofloxacin |
| cm……. | Centimeter |
| Conc……. | Concentration |
| EP…… | European Pharmacopoeia |
| ESI…….. | Electro Spray Ionization |
| FDA…….. | Food and Drug Administration |
| Fig…… | Figure |
| FT-IR…… | Fourier Transform Infra-red |
| g/mol…….. | Grams per Mole |
| GC……. | Gas Chromatography |
| GC-MS……. | Gas Chromatography-Mass Spectrometry |
| $H_2O_2$……. | Hydrogen Peroxide |
| HCl……. | Hydrochloric Acid |
| HPLC……. | High Performance Liquid Chromatography |
| HPTLC…….. | High Performance Thin Layer Liquid Chromatography |
| ICH…… | International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use |
| ID…… | Internal Diameter |
| IP…… | Indian Pharmacopoeia |
| IR…… | Infra-Red |
| LC…….. | Liquid Chromatography |
| LC-MS…… | Liquid Chromatography – Mass Spectrometry |

| | |
|---|---|
| LDA…… | Acid hydrolytic Degradant of LEV |
| LDO…… | Oxidative Degradant of LEV |
| LEV…… | Levofloxacin |
| LOD…… | Limit of Detection |
| LOQ…… | Limit of Quantification |
| µ..... | Micron |
| m/z……. | Mass to Charge Ratio |
| mg…… | Milligram |
| µg…… | Microgram |
| min…….. | Minute |
| mL…….. | Milliliter |
| mL/min…… | Millimeter per minute |
| mM……. | Millimolar |
| mm……. | Millimeter |
| MOX……. | Moxifloxacin |
| N…… | Normality |
| N…… | Number of theoretical plates |
| NA……. | Not available |
| NaOH…… | Sodium Hydroxide |
| NLT…….. | Not Less Than |
| nm……. | Nanometer |
| NMR……. | Nuclear Magnetic Resonance |
| NMT……. | Not More Than |
| NOR……. | Norfloxacin |
| ODS…….. | Octadecyl-Silica |
| OFL…….. | Ofloxacin |
| OPA…… | Ortho Phosphoric Acid |
| PDA…… | Photo Diode Array Detector |
| pKa…… | Dissociation Constant |
| PS……. | Peak Symmetry |
| R & D…….. | Research and Development |
| RH…… | Relative Humidity |
| RRT……. | Relative Retention Time |
| Rs……. | Resolution |
| RSD……. | Relative Standard Deviation |
| RT…… | Room Temperature |
| $R_t$…… | Retention Time |
| SD……. | Standard Deviation |
| SIAM…… | Stability Indicating Assay Method |
| THF…….. | Tetrahydrofuran |
| TLC…….. | Thin layer chromatography |
| USP…… | United States Pharmacopoeia |

| | |
|---|---|
| UV……. | Ultra Violet |
| v/v……. | Volume by Volume |
| W h/$m^2$…… | Watt-hour per square meter |
| w/v…… | Weight by Volume |
| w/w…… | Weight by Weight |
| WHO……. | World Health Organization |
| λ…….... | Lambda (wavelength) |
| $\lambda_{max}$……. | Wavelength at which absorbance is maximum |

# *ABSTRACT*

# Abstract

*Analysis of pharmaceuticals is vital at every stage of development, through the entire journey that starts with synthesis of a new molecule, and continues through various stages of development, until it is used in the patient. Pharmaceutical Analysis, previously with conventional wet chemistry methods and most recently with sophisticated instrumental techniques, assures the quality of the product by ensuring acceptance of the product meeting standards of safety and efficacy. At times, these techniques may need to identify and quantify the drug in presence of other matter, like impurities or degradants, that coexist due to synthesis process involved or arising due to degradation. The testing process needs to be sensitive and specific to establish the content of API in presence of these impurities and degradants and is also not expected to initiate or promote formation of degradants through conditions that are most conducive to degradation.*

*Stability studies meet the objectives of establishing shelf-life and predicting specific conditions for drug degradation. The outcome for such studies provides inputs for adopting good practices that inhibit degradation during the analysis. Application of such processes that promote evolution of degradants could lead to misleading results.*

*The objectives made for the study involved optimization of the experimental conditions that ensures structural integrity to remain secure and also design an algorithm for prediction of retention with molecules that possess identical or similar structural templates.*

*To meet the objectives, a set of drugs from a single class that has fair potential to get affected by experimental conditions commonly adopted during analysis like Fluoroquinolones (FQs) were considered. The drug candidates chosen for the study were*

*based on certain pre-set criteria like chemical structure, physicochemical properties and spectrum of activity- ciprofloxacin (CIP), levofloxacin (LEV), moxifloxacin (MOX), norfloxacin (NOR) and ofloxacin (OFL).*

*Diversity of literature published methods were first assessed with laboratory experiments. Newer HPLC methods were developed, optimized and validated that could be successfully applied for the drugs bearing common structural templates. Additional LC methods were developed to resolve drug peak from other peaks that were found on application of certain conditions that molecules are exposed to during analysis. LC-MS method was useful in assessing the nature of the degradant that accompanied LEV. The proposed optimized experimental conditions for the analysis of FQs of research interest have not yielded any degradants and is recommended for routine analysis.*

*From the data mine representing the experimental variables of the LC analysis of FQs of research interest, multiple linear regression (MLR) models were designed wherein the reported retention times were mapped to various parameters representing molecular descriptors. These models could predict the retention time of compounds. The prediction capability of these models was assessed and statistically validated through application of ANOVA.*

*Keywords: RP-HPLC, fluoroquinolones, stability, ICH, retention time, multiple linear regression models.*

# *CHAPTER 1*

## *INTRODUCTION*

# 1. Introduction

## 1.1.Scope of Pharmaceutical Analysis

Pharmaceutical products are essential commodities of utmost importance, saving lives and alleviating human suffering to say the very least. It is therefore imperative that these medicaments have to meet standards of safety and efficacy in order to fulfil their purpose. To ensure this, every drug and its product has to undergo testing or analysis to check for compliance with quality specifications.

Analysis of a drug is inevitable at every stage of its evolvement, right from synthesis, preclinical and clinical studies, formulation development to finished product quality control. It encompasses qualitative (identification) as well as quantitative evaluation (assay, content uniformity). Synthesis of a new molecule is almost immediately followed by testing methods to establish its yield and purity. Molecules showing promising pharmacological effects are taken for preclinical and clinical trials necessitating analytical methods with satisfactory performance in biological matrices. Formulation development involves choice of excipients and establishing compatibility through various analytical techniques like DSC.

Many times, testing involves detection and quantitation of synthesis related impurities and degradants in trace amounts; or accurate, selective and specific analysis of the molecule of interest, in presence of such related substances.

Impurities are substances that decrease the purity of substances by their presence in the substance. Any substance other than the drug being analysed will thus be an impurity and its mere presence will affect (increase or decrease) the efficiency/ efficacy of the drug, even if this “foreign” substance is not toxic or harmful in itself. It may interact with drug

or excipients and cause harm or decrease effect of the API thereby having profound effects on the health and safety of patients.

**Degradants:** These are impurities produced when the drug degrades to a different chemical entity. Instead of degrading or breaking down, the drug may undergo a chemical reaction and form a derivative. These too, like degradants, may or may not be pharmacologically active, and may decrease the efficacy in spite of being inactive.

**Impurities:** Impurities also include synthesis-related or process-related impurities which maybe by-products introduced during the synthesis of the drug, residual solvents, excess reactants left over due to incomplete conversion, and impurities may even originate from impure reactants used during synthesis. They may be formed during the formulation process, if process variables are not matched to stability profiles of the drug.

A known impurity, 1-(2,6-dichlorophenyl) indolin-2qone, is formed during terminal sterilization by autoclaving in the production of parenteral diclofenac sodium. Diclofenac undergoes intramolecular cyclic reaction at autoclaving temperatures, producing the impurity in quantities beyond the limit prescribed for it[1].

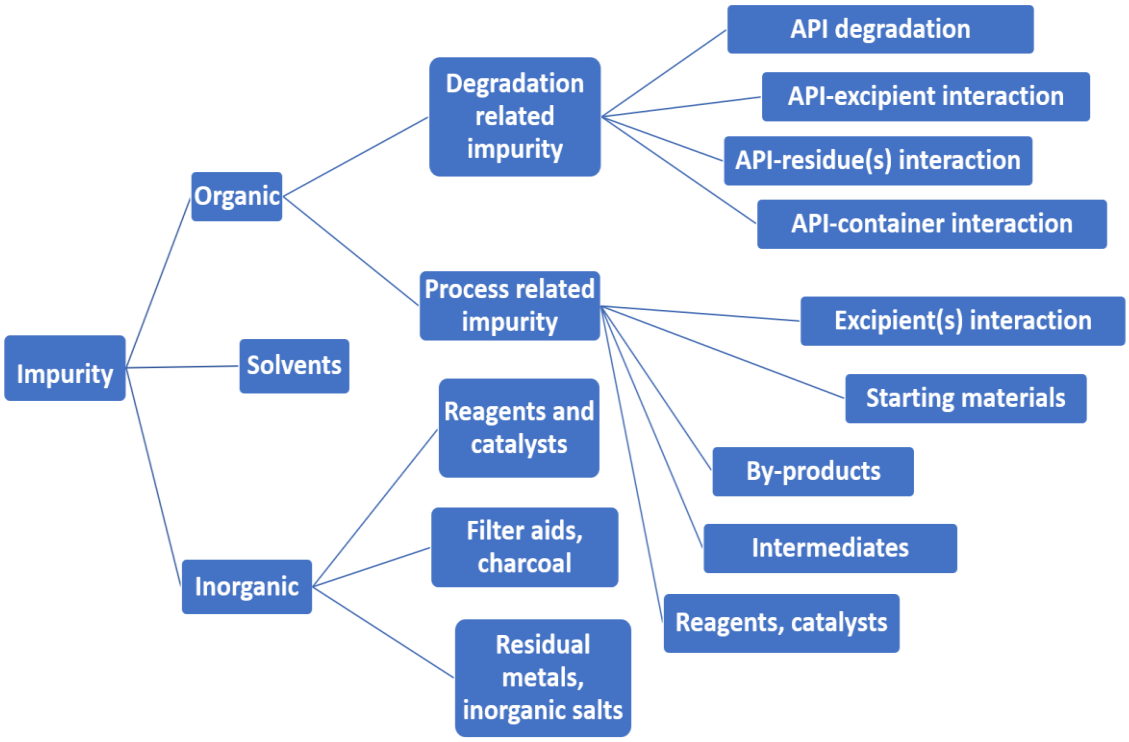


**Fig.1.1. Types of Impurities**

**Related Substances:** Impurities which are most regularly/ frequently associated with a drug are termed as related substances and find mention in the monographs of the drug in official compendia, often accompanied by analytical methods to detect them.

### 1.2. Analytical Method Development

A systematic approach to development of an analytical method involves certain steps which ensure the applicability and suitability of the proposed method. The analytical method comprises not just the actual detection or determination procedure, but encompasses additional or auxiliary procedures such as sample preparation which may range from simple dilution to complex extraction procedures from biological matrices.

Before starting the development process, the developer has to gather all available data related to the analyte and characterize the substance with respect to its physical and chemical properties, solubility being one of the important properties to be considered. While doing this the developer has to gain a clear outlook towards the purpose of method development, whether one is aiming at developing a stability indicating method, type and extent of degradation the method is expected to detect and quantify and the extent of sensitivity and range desired in the method. With this in mind, literature is searched thoroughly for existence of similar methods which could possibly be adopted as such or adapted through some modifications. If this survey establishes the need for development of a new analytical method, one can then proceed with the next stage equipped with data from literature search and select the analytical technique best suited for needs already outlined. This is then followed by the instrumental setup and initial studies/ trials. The results of the trials are evaluated with system suitability parameters established for the chosen analytical technique. A trial showing promising results may then be chosen for further optimization wherein each method related variable (e.g., pH of mobile phase and

its composition and flow rate in case of chromatography) is optimized. Once optimized the method needs to be tested on marketed preparations and checked for recovery of spiked samples. The developed method is taken up for validation which happens almost simultaneously with the development process.

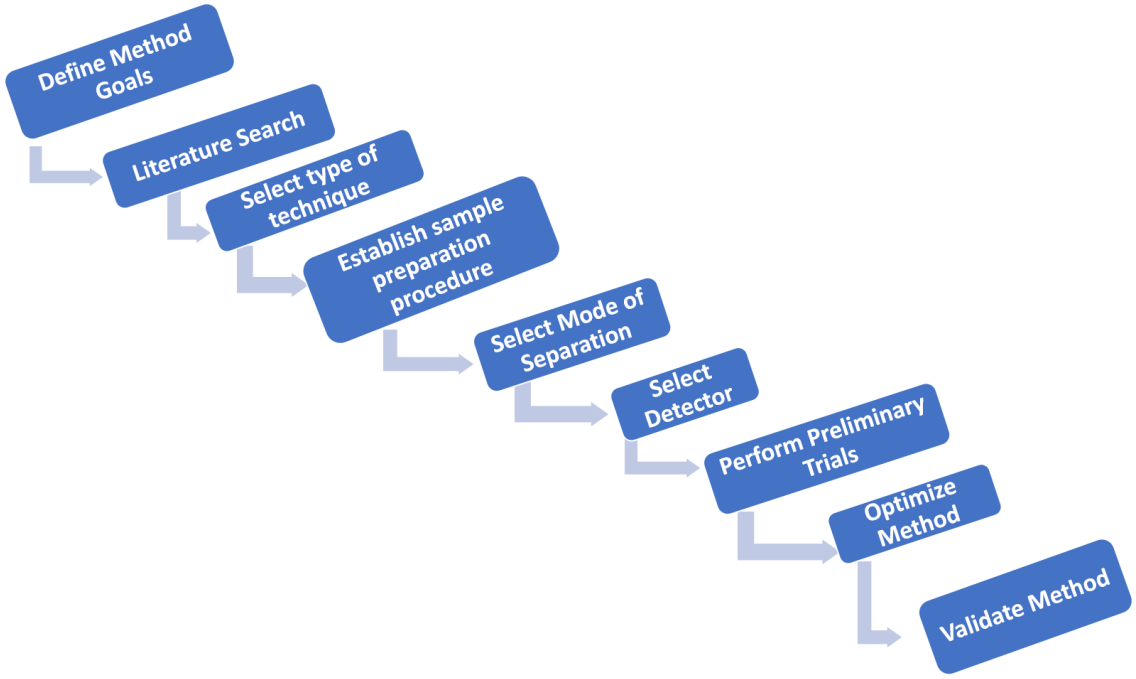


**Fig.1.2. Analytical Method Development**

While developing new methods, the conditions that are conducive to degradation of drug under study have to be avoided. For this purpose, the stability profile of the drug candidate has to be investigated through a literature search or experimentally.

### 1.3. Method Validation

To ensure the quality of a developed method, a step-by-step process called validation is applied to determine whether the analytical method fulfills the needs for the intended laboratory purpose, validation primarily involves a number of parameters. Before being used in analytical laboratories, all developed analytical procedures must be validated and revised till satisfactory.

According to different guidelines, validation is defined as follows:

**FDA-guidelines:** Validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes[2].

**EU-guidelines:** Action of proving, in accordance with GMP-principles that any procedure, process, equipment, material, activity or system actually leads to the expected results[3].

**ICH-guidelines:** Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use[4].

**Necessity of Method Validation Studies:**

- To measurably specify the system performance.
- To identify and quantify potential for error.
- To recognize differences between each method.
- To assure adherence to regulatory guidelines.

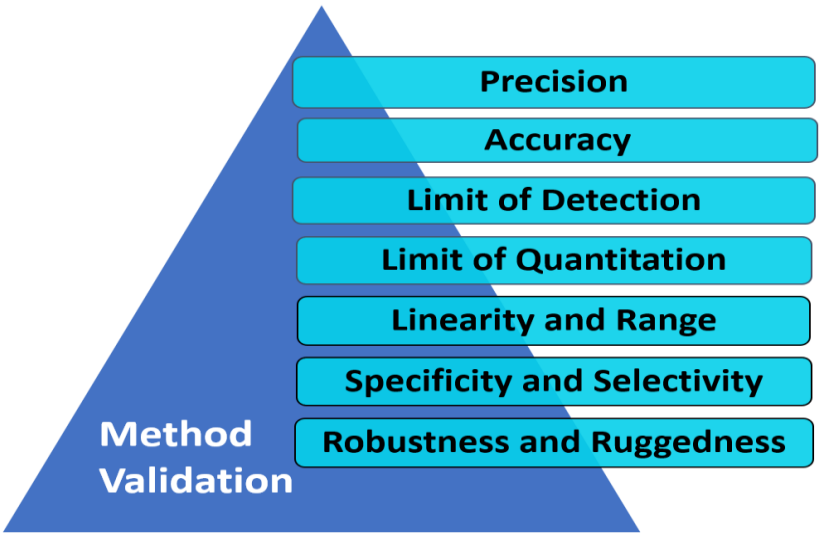


**Fig.1.3. Method Validation**

**Parameters for Method Validation**

Specificity, selectivity, accuracy, repeatability, intermediate precision, reproducibility, accuracy, range, LOD, LOQ, robustness, and ruggedness are the primary parameters listed by many authorities, organizations, and the ICH guidelines.

### 1.4 Stability Studies and Stability Indicating Analytical Methods

Stability Indicating Analytical Method (SIAM) is an analytical method with the additional capacity of ensuring detection of instability. It is proved through an extended part of validation of the assay process. In the world of pharmaceuticals, it is necessary to ensure safety as well as efficiency of drug substance and its product. The ICH and FDA guidelines state the necessity of maintaining, assessing, and documenting the stability profile of drugs[5].

Stability studies must be performed before registering a new medicine and long-term 12-month studies as well as 6-month accelerated stability studies are required for this purpose. Intermediate stability investigations can be milder than accelerated studies, but such studies are essentially time consuming. Forced degradation studies, on the other hand, take less time and yield degradation products faster (possibly in a few hours). The purpose of these stability studies is mainly to:

a. Determine intrinsic stability of drug under study.
b. Establish degradation pathways for the drug.
c. Identify degradation products and distinguish them from process-related impurities.
d. To identify suitable physical state for dosage form, establish compatibility with excipients and study the protective or catalytic effect of excipients on degradation of drugs in formulations.
e. To select appropriate manufacturing process and packaging strategy to maintain stability of the drug in final dosage form.
f. To predict and prolong shelf-life of the drug and its products.
g. Validate stability indicating capability of testing method.

h. Aid in developing a rational analytical method that does not in itself promote degradation in the drug by adopting suitable test parameters.

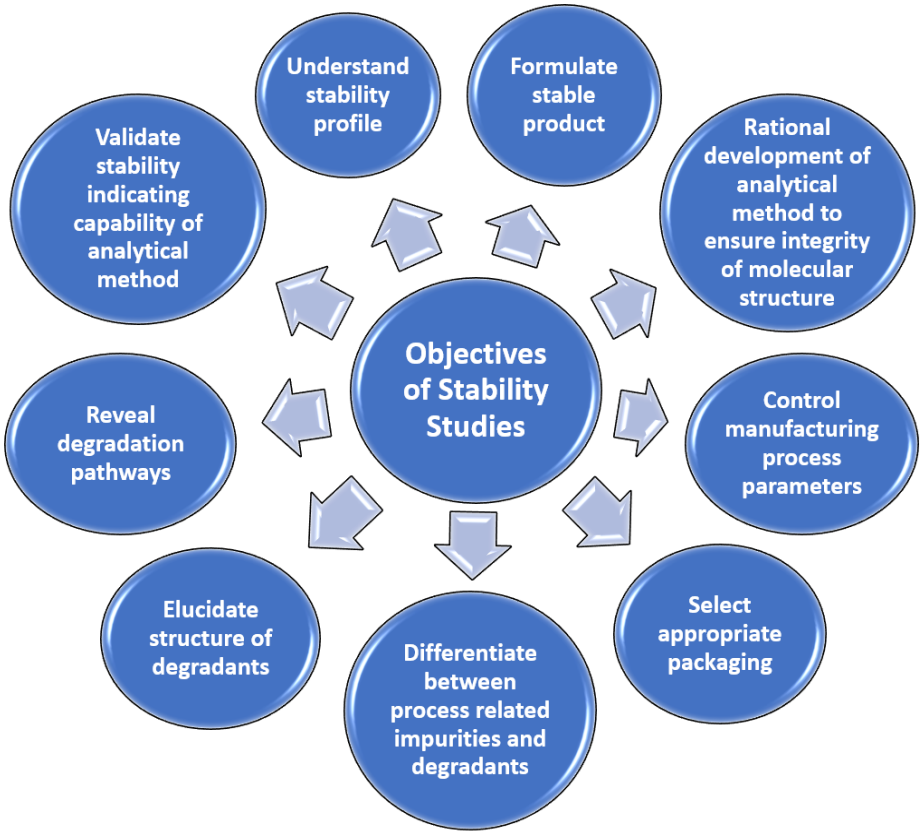


**Fig.1.4. Objectives of Stability Studies**

International Conference on Harmonization (ICH) emphasizes forced degradation studies to develop stability indicating assay methods (SIAM) under various stresses such as oxidation effect, pH, light exposure, exposure to moist and dry heat. In forced degradation studies focus is on getting maximum possible degradation products which entails use of extreme stress not encountered in routine analysis. This could possibly lead to production of unusual degradants and misleading results.

One of the guidance documents, Q1A (R2) – Stability Testing of New Drug Substances and Products, states: “Stress testing is likely to be carried out on a single batch of the drug

substance. The testing should include the effect of temperatures (in 10°C increments (i.e., 50°C, 60°C) above that for accelerated testing, humidity (i.e., 75% relative humidity or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension."

Stressed study samples are analysed using titrimetry, spectrophotometry, and chromatography. Currently, chromatography and spectrophotometry in combination are utilised to separate and identify degradants. Use of such hyphenated techniques, including LC-MS and GC-MS, reduces study time and offers more accurate qualitative and quantitative degradation data.

Chromatography is versatile for component separation and different stationary phases, mobile phases, and detecting technologies enable varied analyses. HPLC and HPTLC are widely used because of their great resolution, sensitivity, and specificity.

The ICH recommendation recognizes that strict deterioration rules are not possible and permits some flexibility in application of stress conditions. Accelerated stability research and scientific understanding of the product's breakdown mechanism under typical use situations should guide forced degradation conditions.

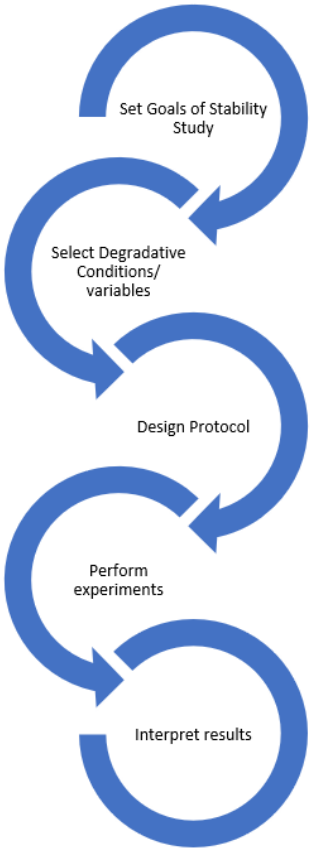


**Fig.1.5. Schematic Strategies for Plan of Stability Studies**

A suitable strategy for stability studies may be devised after mapping the available data from literature to the goals of research. Minimal conditions to be considered for forced degradation studies are:

1. **Hydrolysis**: By observing the stability of the medication in 0.1 N HCl (or $H_2SO_4$) or 0.1 N NaOH, the hydrolytic breakdown of a drug molecule in acidic and alkaline conditions can be investigated. Testing can be terminated at the step where a reasonable amount of degradation is shown, but the sample should be refluxed in stronger acid or alkali for a longer period of time if no degradation is observed under mild circumstances. Alternately, if 100% degradation occurs after exposing the

medications to the initial mild conditions, acid/alkali strength and reaction temperature can be lowered.

**Table 1.1. Drugs undergoing Hydrolytic Degradation**

| Drug | Conditions | Remarks | Reference |
|---|---|---|---|
| Ranitidine | 1N NaOH, boiling for 20 min | Drug degraded by 84.4% | [6,7] |
| Retinoic acid | 0.1N HCl, refluxing for 5min | 65% recovery of drug | [6,7] |
| Omeprazole | 1N $H_2SO_4$, boiling for 5min | Total degradation | [7,8] |

2. **Thermal Degradation (dry heat and moist heat):**

Dry heat: Stress tests for dry heat degradation can be performed by heating the drug in solid form at a higher temperature in an oven.

Moist heat: By maintaining the drug in solution at 50°C and possibly 75% relative humidity for three months in a humidity chamber, moist heat degradation can be examined. Effect of higher temperatures and humidity conditions can also be studied.

**Table 1.2. Drugs undergoing Thermal Degradation**

| Drug | Conditions | Remarks | Reference |
|---|---|---|---|
| Luliconazole | 80°C, 2h of dry heat | 14% degradation | [9] |
| Teneligliptin | 69°C, 48 h, in methanol | 29.21% degradation | [10] |
| Favipiravir | 70°C, 24h of dry heat | 20.76% degradation | [11] |

3. **Oxidation**: It has been advised to utilize hydrogen peroxide in the concentration range of 3 to 30% to test for oxidation. When exposed to 3% hydrogen peroxide at ambient temperature for a very limited period of time, some medications experience

substantial deterioration. While in some other cases, even the most extreme circumstances, and an exposure to large concentrations of hydrogen peroxide does not result in any appreciable deterioration. This obviously indicates that the drug is stable to oxidative degradation.

**Table 1.3. Drugs undergoing Oxidative Degradation**

| Drug | Conditions | Remarks | Reference |
|---|---|---|---|
| Ranitidine HCl | 3% $H_2O_2$, at RT in 20min | 37.8% loss in potency | [6,7] |
| Mefenamic acid | 3% $H_2O_2$, at RT in 16h | 7.79% degradation | [12] |
| Loperamide | 1.5% $H_2O_2$, immediately | 3.2% of the cis N-oxide of loperamide and 2.4% of the trans N-oxide | [7,13] |

4. **Photolysis**: Many drugs undergo photodegradation by various pathways dependent on molecular structure and light conditions they are exposed to. Norfloxacin undergoes photodehalogenation as well as photoinduced ring cleavage[14]. While studying photostability, there are numerous parameters that need to be taken into account, such as the source of light, the duration of exposure, and the sample state. For the light source, use of lamps emitting only in the UV region, including UV-C, exposure of study sample on window sill and exposure to window-glass filtered daylight has been reported[15]. Conditions closest to 'in use' conditions need to be chosen[16].

   UV light: Photolytic experiments should involve exposure to light with either a cool white or UV fluorescent lamp combo. Fluorescent light exposure energy must be at least 1.2 million lux hours, and if decomposition is not observed, intensity must be

increased by a factor of five. The substance can be deemed photo stable if there has been no further breakdown[17].

Sunlight: Photolytic investigations ought to cover how long medication solutions are exposed to the sun. A few hours to several months of exposure time are possible and studies on photolysis are generally conducted at room temperature[5].

**Table 1.4. Drugs undergoing Photolytic Degradation**

| Drug | Conditions | Remarks | Reference |
|---|---|---|---|
| Nifedipine | Visible/UV light, 30min on TLC plate | Converted to nitroso derivative within 5-30min, no intact drug left after 5h | [6,7] |
| Retinoic acid | 254nm, 20cm distance, 2h | 50% degradation | [18] |
| Sodium Levothyroxine | Intact tablets, longwave UV, 168h | 91.9 % drug recovery | [19] |

### 1.5 Appropriateness of Developed Analytical Methods

The analytical methods need to be strong and sensitive enough not to skip upon any other additional component in test substance, and at the same time reasonably harmless on the molecular structure, imperatively the conditions used during testing should not by themselves initiate any degradation of the molecule under test. Often during method development and optimization, molecules are subjected to severe conditions of stress leading to a compromise in the integral structure of the molecule resulting in the formation of degradants regarded as impurities.

The undesirable substances co-existing with main component could be process related impurities or degradants formed due to an unfavorable or hostile process unassumingly applied due to inadequate information on the stability profile of the drug. Such degradants

would not individually get detected by erstwhile less sensitive analytical methods like volumetric and gravimetric analysis as their minimum detectable concentrations are much higher than the advanced analytical techniques.

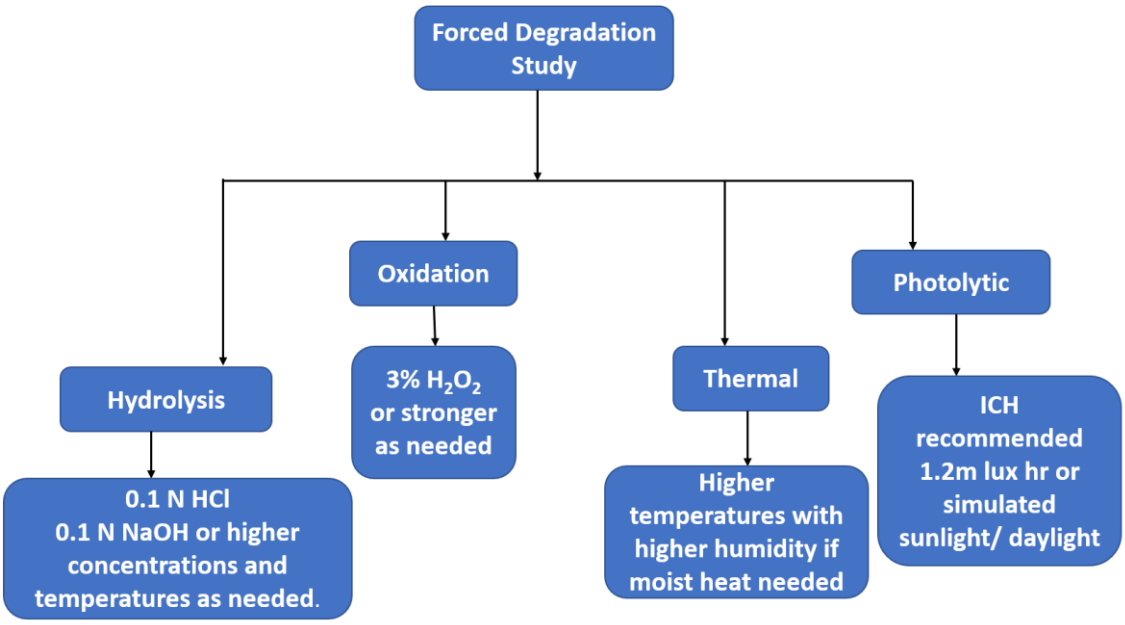


**Fig.1.6. Nature of Forced Degradation Studies**

Process related impurities are predictable and can be prevented in the drug substance. Presence of degradants can also be envisaged when the drug is exposed to adverse conditions during testing like hydrolysis, oxidation, higher temperature and light. If the structure is susceptible to degradation under applied experimental conditions, caution needs to be exercised during the design of new method development and measures taken for control of deterioration of drug even during the process of testing. Issues pertaining to origin of impurities during the testing process have been a major concern in drug analysis and need to be addressed authoritatively to ensure uncertainties while drawing inferences with regard to purity of the drugs.

Ranitidine which thermally degrades to carcinogenic N-nitrosodimethylamine (NDMA) is a typical example of drugs undergoing degradation during analysis and hence Gas chromatographic analysis was found to be inappropriate for analysis of NDMA in ranitidine[20]. On account of this in 2019, many reputed brands had to be recalled from

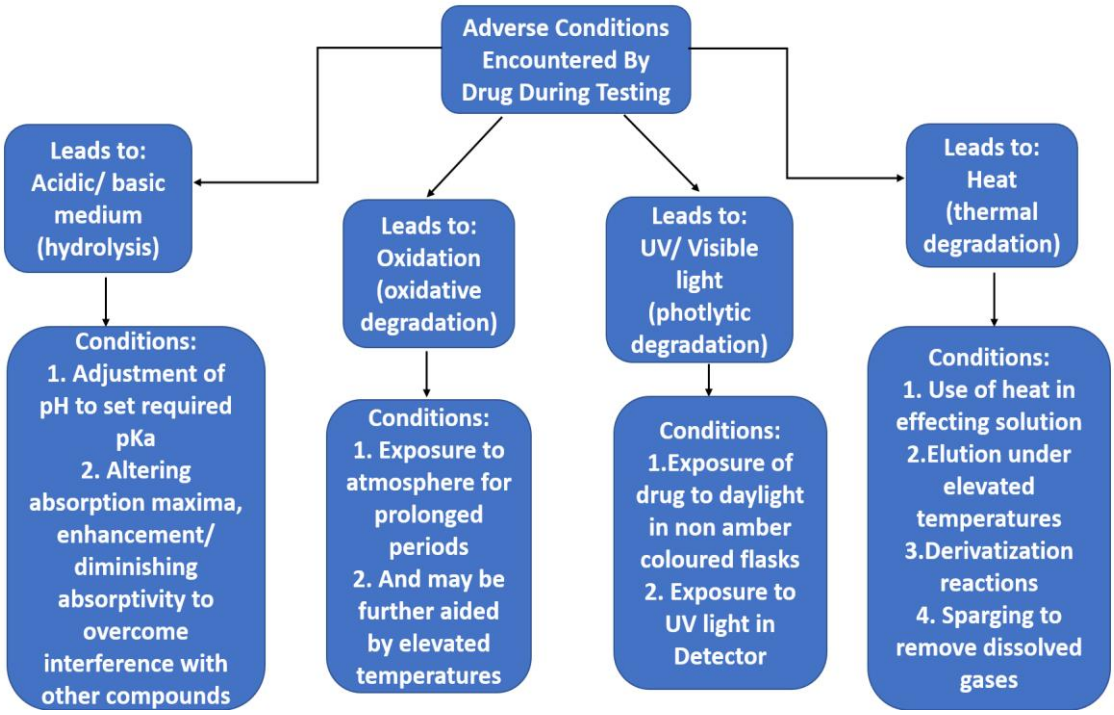


**Fig.1.7. Adverse Conditions Encountered by Drug during Testing**

the market and USFDA later developed a simulated gastric fluid model to be used with LC-MS to investigate the relevance in biological and in vivo environments since GC testing method was found unsuitable[21–23].

Quinolin-8-yl 1-pentyl-(1*H*-indole)-3-carboxylate (QUPIC), which is a synthetic cannabinoid, was found to undergo thermal degradation during gas chromatography–mass spectrometry (GC–MS), probably because of the presence of an ester bond in its structure, the thermal degradation being more pronounced when the drug was dissolved in methanol or ethanol. The degradation was seen to be less when nonalcoholic solvents

such as acetone and chloroform were used. In such cases, the effects of various parameters, such as injection methods (splitless or split, and split ratio), injector temperatures, and injector liners on the thermal degradation of drugs needs to be studied minutely while developing methods for analysis[24]. Another drug, α-Pyrrolidinopentiophenone (α-PVP), a popular recreational drug in Japan, has also been reported to undergo thermal degradation during GC-MS analysis[25].

Studies revealed that clozapine-N-oxide, which is the principal urinary metabolite of the antipsychotic agent clozapine, interferes with clozapine during analysis by GC-MS since significant on-column reduction of clozapine-N-oxide to the parent drug occurred during analysis. Hence sample preparation techniques need attention to avoid favorable conditions like in this particular case, the co-extraction of N-oxide led to artifactual contribution of this metabolite in the detection of clozapine[26].

Analytical methods like TLC also demonstrate event of degradation as seen in the case of phenylbutazone and its products that need special precautions for minimizing on-plate oxidation[27].

Certain ***pseudo degradation*** products have also been observed during stress degradation studies. These are not formed through degradation of the drug under study, but they are products of reaction of the drug with co-solvent and/ or stressor reagent used in the stress reaction mixtures. These unwanted reactions need to be avoided in order to prevent false interpretations of results. Stress testing on tenofovir disoproxil fumarate using methanol as solvent produced two pseudo degradants reported to be methyl esters of the drug. Certain other cases involve chlorination of compounds like efavirenz and ivabradine in the presence of hydrochloric acid that incidentally was used as a stress reagent [28,29].

In order to avoid contributing to already existing impurities and giving false results, testing methods that induce formation of new impurity/ and or potentiate the concentration of a previously existing impurity should not be proposed.

Rationale of analytical method development is not limited only to the design of process that promotes selective and sensitive detection and determination of all the components in test samples, but also assures safe medium or environment to enable molecules to retain their integrity and not contribute to synergism of impurities leading to misinterpretation.

# *CHAPTER 2*

## *RESEARCH ENVISAGED*

## 2. Research Envisaged

The process of sensible analytical method development requires an expertise with regard to knowledge of chemistry of molecules as well as understanding of its behavior in the environment that it is exposed to during testing. There have been several evidences published wherein the molecules under test have behaved indifferently and contributed to evolution of undesirable compounds leading to serious errors while drawing conclusions.

The properties exhibited by molecules, generally a reflection of its composition, could be exploited for rational method development that could ease the process and ensure accuracy and reliability in the results. Also, tapping the right locations in the molecule could unlock assumptions of extending methods to such other molecules bearing identical templates in their structure.

Analytical methods are generally based on specific chemical structure of the analyte that drives its chemical and physicochemical properties. This study envisages the possibility of developing methods that could be successfully used for analysing several fluoroquinolones with minor modification in the process. The proposed optimized methods upon validation shall be extended for assessment of their strength with regard to protection of the Fluoroquinolone against extended degradation during the course of analysis. Also, the methods would be evaluated for their stability indicating capabilities.

The study data will be used along with the one recorded from literature to identify the factors that govern the retention profile of the molecules. Retention of a molecule expectedly on the basis of its polarity – a physicochemical parameter could be explored

for possible sync through development of appropriate algorithms using published data from reputed literature.

The aims and objectives (primary and secondary) of the project work are listed below:

- ✓ To develop HPLC methods with isocratic elution involving common mobile phase for analysis of selected fluoroquinolones and extend them for analysis in formulations.
- ✓ To investigate stability profiles of selected fluoroquinolones under stressed conditions involving hydrolysis, temperature, light and oxidation.
- ✓ To develop models for prediction of retention times of molecules using appropriate molecular descriptors and statistical regression models.

Among the objectives listed the primary and secondary objectives of the research are defined as follows:

**Primary Objectives**

- To develop versatile HPLC methods with softer operation parameters extendable to a wider range of Fluoroquinolone class of drugs.
- To identify appropriate factors that affect retention profile of compounds for design of regression models for prediction of Rt in fluroquinolones and to extend application of regression model for prediction of retention time for other related compounds.

**Secondary Objectives**

- To investigate the applicability of compendial methods of analysis upon extension to other fluroquinolones.
- Review methods reported for analysis of selected fluroquinolones and study the diversity of variables applied for separations in special cases with co-existing compounds or formed due to stress conditions.
- To investigate stability profiles of selected fluoroquinolones under stressed conditions involving hydrolysis, temperature, light and oxidation.

# *CHAPTER 3*

# *REVIEW OF LITERATURE*

# 3. Review of Literature

Envisaging principal objectives of study, a comprehensive literature survey was undertaken to familiarize different methods published on various fluoroquinolones (FQs) as this series of molecules provides large number of molecules under antibacterial class with diverse functional constituents in their structures. Also, an elaborate literature on analytical methods is accessible to generate a data mine with regard to operational variables like mobile phase composition, pH, stationary phase and so on that could assist in the prediction study of retention profiles.

## 3.1 Compendial Methods

While reviewing the compendial methods of analysis for FQs of research interest and their finished dosage forms, diverse methods have been recommended that extend over a wide range from potentiometric non-aqueous volumetry to more complex RP-HPLC. In spite of compounds showing similarity in their chemical structures, methods for their analysis are widely different and attracts rationalization. Although HPLC methods recommend use of C18 columns, phenylsilyl columns are recommended in certain procedures (moxifloxacin).

Rationalization of analytical method for application to a specific chemical class that encompass a large number of molecules could be an attempt to seek seamless exploration and their application for accurate analysis of existing or new compounds. This could prove beneficial for routine analysis of pharmaceuticals especially to pharma companies dealing with large number of batches of multiple products belonging to specific chemical class.

## PHARMACOPOEIAL DATA ON ASSAY OF SELECTED FLUOROQUINOLONES

**Table 3.1 Assay Methods for Selected FQs and their marketed products**

| DRUG / formulation | IP 2014 to 2022[30] | BP 2013 to 2023[31] | USP 36 (2013) to USP 44- NF 39 2021[32] |
|---|---|---|---|
| CIP pure drug | HPLC<br><br>MP: ACN and Buffer (13:87)<br>Buffer: 0.025M phosphoric acid adjusted to pH 3.0 with TEA<br>C18 (25 cm x 4.0 mm) at 30 °C;<br>1.5 mL/min<br>278 nm | Potentiometric titration; non-aqueous using perchloric acid | HPLC<br><br>MP: ACN and Buffer (13:87)<br>Buffer: 0.025M phosphoric acid adjusted to pH 3.0 with TEA.<br>C18 (25 cm x 4.6 mm) at 30 °C;<br>278 nm;<br>1.5 mL/min |
| CIP HCl | HPLC<br><br>MP: ACN and Buffer (13:87)<br>Buffer: 0.025M phosphoric acid adjusted to pH 3.0 with TEA<br>C18 (25 cm x 4.0 mm) at 30 °C<br>1.5 mL/min<br>278 nm | HPLC<br><br>MP: ACN and Buffer (13:87)<br>Buffer: 2.45 g/L solution of phosphoric acid adjusted to pH 3.0 with TEA.<br>C18 (25 cm x 4.6 mm) at 40 °C;<br>1.5 mL/min<br>278 nm | HPLC<br><br>MP: ACN and Buffer (13:87)<br>Buffer: 0.025M phosphoric acid adjusted to pH 3.0 with TEA.<br>C18 (25 cm x 4.6 mm) at 30 °C;<br>278 nm;<br>1.5 mL/min |
| CIP Injection/ Infusion | HPLC<br><br>MP: ACN and Buffer (13:87) | **HPLC** | HPLC<br><br>MP: ACN and Buffer (13:87) |

| | | | |
|---|---|---|---|
| | Buffer: 0.025M phosphoric acid adjusted to pH 3.0 with TEA<br>C18 (25 cm x 4.0 mm) at 30°C<br>278 nm | **13: 87 (ACN: 0.245 % w/v OPA adjusted to pH 3.0 with TEA;**<br>**C18 (25 cm x 4.6 mm) at 40 °C;**<br>**1.5 mL/min;**<br>**278 nm** | Buffer: 0.025M phosphoric acid adjusted to pH 3.0 with TEA;<br>C18 (25 cm x 4.6 mm) at 30 °C;<br>278 nm |
| CIP Eye Drops (IP)/ Ophthalmic solution (USP) | HPLC<br><br>MP: methanol and Buffer (25:75)<br>Buffer: 0.005 M tetrabutyl-ammonium phosphate, adjusted to pH 2.0 with OPA<br>C18 (25 cm x 4.0 mm) at 30°C<br>280 nm | **HPLC**<br><br>**MP - 13: 87 (ACN: 0.245 % w/v OPA adjusted to pH 3.0 with TEA**<br>**C18 (25 cm x 4.6 mm) at 40 °C;**<br>**1.5 mL/min;**<br>**278 nm** | **HPLC**<br><br>**MP: ACN and aqueous (12:88)**<br>**Aqueous: 0.29 % v/v, adjusted to pH 5.2 with TEA.**<br>**C18 (25 cm x 4.6 mm, 5µ) at 39°C,**<br>**1 mL/min;**<br>**278 nm** |
| CIP Tablets | HPLC<br><br>MP: ACN and Buffer (13:87)<br>Buffer: 0.025M phosphoric acid adjusted to pH 3.0 with TEA<br>C18 (25 cm x 4.0mm) at 30°C<br>278 nm | **HPLC**<br><br>**13: 87 (ACN: 0.245 % w/v OPA adjusted to pH 3.0 with TEA**<br>**C18 (25 cm x 4.6 mm) at 40 °C;**<br>**1.5 mL/min;**<br>**278 nm** | HPLC<br><br>MP: ACN and Buffer (13:87)<br>Buffer: 0.025M phosphoric acid adjusted to pH 3.0 with TEA.<br>1.5 mL/min<br>C18 (25 cm x 4.6mm) at 30°C;<br>278 nm |
| CIP Ophthalmic Ointment | NA | NA | **HPLC**<br><br>**MP: ACN and aqueous (12:88)** |

| | | | |
|---|---|---|---|
| | | | **Aqueous: 0.29 % v/v, adjusted to pH 5.2 with TEA.**<br>C18 (25 cm x 4.6 mm, 5µ) at 39°C,<br>1 mL/min;<br>278 nm |
| CIP Ear Drops | | **HPLC**<br><br>**MP - 13: 87 (ACN: 0.245 % w/v OPA adjusted to pH 3.0 with TEA**<br>**C18 (25 cm x 4.6 mm) at 40 °C;**<br>**1.5 mL/min,**<br>**278 nm** | |
| CIP oral suspension | | | **HPLC**<br><br>**MP: Methanol and Solution A (20:80)**<br>**Solution A: 13.6 g of sodium acetate in 1000 mL**<br>**of water and 1 mL of TEA. Adjust with**<br>**phosphoric acid to a pH of 2.0.**<br>**C18 (15 cm x 4.6 mm, 5µ) at 40°C,**<br>**1.2 mL/min;**<br>**278 nm** |
| LEV pure drug | **HPLC** | Potentiometric titration; non-aqueous using perchloric acid | HPLC |

| | | | |
|---|---|---|---|
| | **MP: Methanol and Buffer (3:7)**<br>**Buffer: 8.5 g/L of ammonium acetate, 1.25 g/L of cupric sulfate, pentahydrate, and 1.3 g/L of L-isoleucine in water.**<br>**C18 (15 cm x 4.6 mm) at 45°C;**<br>**360 nm** | | MP: Methanol and Buffer (3:7)<br>Buffer: 8.5 g/L of ammonium acetate, 1.25 g/L of cupric sulfate, pentahydrate, and 1.3 g/L of L-isoleucine in water.<br><br>C18 (15 cm x 4.6 mm) at 45°C;<br>360 nm |
| LEV infusion and LEV injection and LEV tablets | HPLC<br><br>MP- ACN: 0.05M citric acid monohydrate: 1M ammonium acetate (15:84:1)<br>C18 (25 cm x 4.6 mm) at 30 °C | **HPLC**<br><br>**MP: 0.0874 % w/v copper sulphate pentahydrate, 0.091% w/v isoleucine and 0.594% w/v ammonium acetate in a mixture containing 3 volumes of methanol and 7 volumes of water.**<br>**C18 (25 cm x 4.6 mm) at 45 °C;**<br>**360 nm**<br>**0.8 mL/min** | **HPLC (Tablets)**<br><br>**MP: 0.0874 % w/v copper sulphate pentahydrate, 0.091% w/v isoleucine and 0.594% w/v ammonium acetate in a mixture containing 3 volumes of methanol and 7 volumes of water.**<br>**C18 (25 cm x 4.6 mm) at 45 °C;**<br>**360 nm**<br>**0.8 mL/min** |
| LEV oral solution | NA | NA | HPLC<br><br>MP: Acetonitrile and water (18:82) with 0.1 % trifluoroacetic acid |

| | | | |
|---|---|---|---|
| | | | Phenylsilyl column (25 cm x 4.6 mm) at 30°C;<br>294 nm |
| LEV Eye drops | | **HPLC**<br><br>**MP: 0.0874% w/v copper sulphate pentahydrate, 0.091% w/v isoleucine and 0.594% w/v ammonium acetate in a mixture containing 3 volumes of methanol and 7 volumes of water.** | |
| MOX HCl | HPLC<br><br>MP: Methanol and buffer (28:72)<br>Buffer: 0.05% tetrabutylammonium hydrogen sulphate, 0.1% $KH_2PO_4$, 0.34% OPA<br><br>Phenylsilyl column (4.6 mm x 25 cm) at 45 °C | HPLC<br><br>MP: Methanol and buffer (28:72)<br>Buffer: 0.05% tetrabutylammonium hydrogen sulphate, 0.1% $KH_2PO_4$, 0.34% orthophosphoric acid<br><br>Phenylsilyl (4.6 mm x 25 cm) column at 45 °C | HPLC<br><br>MP: Methanol and buffer (28:72)<br>Buffer: 0.05% tetrabutylammonium hydrogen sulphate, 0.1% $KH_2PO_4$, 0.34% orthophosphoric acid<br><br>(4.6 mm x 25 cm) column at 45 °C; 0.9 mL/ min |
| MOX parenteral | | **HPLC**<br><br>**MP: Methanol and buffer (28:72)**<br>**Buffer: 0.05% tetrabutylammonium hydrogen** | |

| | | | |
|---|---|---|---|
| | | **sulphate, 0.1% $KH_2PO_4$, 0.34% OPA**<br>**Phenylsilyl (4.6 mm x 25 cm) column at 45 °C** | |
| MOX Eye Drops IP/ Ophthalmic Solution USP | HPLC Gradient<br><br>MP: Methanol and buffer<br>Buffer: 0.05% tetrabutylammonium hydrogen sulphate, 0.1% $KH_2PO_4$, 0.34% orthophosphoric acid<br>Phenylsilyl column (4.0 mm x 25 cm) at 45 °C | NA | HPLC Gradient<br><br>MP: Methanol and buffer<br>Buffer: 0.05 g% of tetrabutylammonium hydrogen sulfate and 0.1 g% of monobasic potassium phosphate 0.2 % of phosphoric acid<br>Phenylsilyl (4.0 mm x 25 cm) column at 45 °C;<br>293 nm |
| MOX tablets | | **HPLC**<br><br>**MP: Methanol and buffer (28:72)**<br>**Buffer: 0.05% tetrabutylammonium hydrogen sulphate, 0.1% $KH_2PO_4$, 0.34% OPA**<br>**Phenylsilyl (4.6 mm x 25 cm) column at 45 °C** | **HPLC**<br><br>**MP: ACN and Solution A (50:1000)**<br>**Buffer: 1.36 g/L of monobasic potassium phosphate in water. Add 2 mL of TEA for each L of the solution and adjust with phosphoric acid to a pH of 1.9.** |

| | | | |
|---|---|---|---|
| | | | **Solution A: Methanol, *n*-propyl alcohol, and Buffer (300:34:666)**<br>**Phenylsilyl (4.0 mm x 25 cm) column at 45 °C;**<br>**293 nm;**<br>**1.5 mL/min** |
| NOR pure drug | Potentiometric titration; non-aqueous using perchloric acid | Potentiometric titration; non-aqueous using perchloric acid | **HPLC Gradient**<br><br>**MP: ACN and water adjusted with phosphoric acid to a pH of 2.0**<br>**USP L60 (amide) column (4.6 mm x 25 cm) at 60 °C;**<br>**1.4 mL/min;**<br>**265 nm** |
| NOR Eye Drops IP / Ophthalmic solution USP | HPLC<br><br>MP: Methanol and 0.1% orthophosphoric acid (30:70)<br>C18 (3.9 mm x 30 cm) at 50 °C;<br>280nm<br>2 mL/min | NA | HPLC<br><br>MP: ACN and (1 in 1000)dil. phosphoric acid (15:85)<br>C18 (3.9 mm x 30 cm) at 50 °C;<br>278 nm<br>0.5 mL/min |
| NOR Tablets | HPLC | **HPLC** | HPLC |

| | | | |
|---|---|---|---|
| | MP: ACN and 0.1% orthophosphoric acid (15:85)<br>C18 (3.9 mm x 30 cm) at 40 °C;<br>275 nm<br>2 mL/min | **MP: ACN and (1 in 1000) dil. phosphoric acid (15:85)**<br>**C18(3.9 mm x 30 cm) at 40 °C,**<br>**275 nm** | MP: ACN and (1 in 1000) dil. Phosphoric acid (15:85)<br>C18(3.9 mm x 30cm) at 40 °C;<br>2 mL/min;<br>275 nm |
| OFL pure drug | **HPLC**<br><br>**MP: ACN and buffer (20:80)**<br>**Buffer: 27.2 g/L of potassium dihydrogen orthophosphate anhydrous, adjusted to pH 2.4 with OPA**<br>**C18 column (10 cm x 4.6 mm)** | Potentiometric titration; non-aqueous using perchloric acid | Potentiometric titration; non-aqueous using perchloric acid |
| OFL Infusion | HPLC<br><br>MP: ACN and buffer (20:80)<br>Buffer: 6.8 g/L of $KH_2PO_4$, 0.47 g/L sodium 1-hexane sulphonate and 1 ml/L TEA in water, adjusted to pH 3.0 with OPA.<br>C18 (4.6 mm x 25 cm) column | NA | NA |
| OFL Ophthalmic Solution / Eye Drops | HPLC<br><br>MP: ACN and buffer (20:80)<br>Buffer: phosphate buffer pH 7.25 prepared by dissolving 2.54 g/L of | **HPLC**<br><br>**MP: 10 volumes of acetonitrile and 90 volumes of a solution containing 2.72% w/v of** | HPLC<br><br>MP- ACN, 0.24% sodium dodecyl sulphate,<br>and glacial acetic acid (400:580:20) |

| | | | |
|---|---|---|---|
| | tetrabutyl ammonium hydrogen sulphate and 3.56 g/L of disodium hydrogen phosphate in water<br>C18 column (4.6 mm x 15 cm) | **potassium dihydrogen phosphate, previously adjusted to pH 3.3 with orthophosphoric acid.**<br>**C18 (10cm x 4.6 mm, 3.5 µ)**<br>**294 nm; 2 mL/min** | C18 (4.6 mm x 25 cm) at 35 °C; 294 nm; 1.5 mL/min |
| OFL Oral Suspension | HPLC<br><br>MP: ACN and buffer (20:80)<br>Buffer: 27.2 g/L of potassium dihydrogen orthophosphate anhydrous, adjusted to pH 2.4 with orthophosphoric acid<br>C18 column (10 cm x 4.6 mm) | NA | NA |
| OFL Tablets | HPLC<br><br>MP: ACN and buffer (8:92)<br>Buffer: 27.2 g/L of potassium dihydrogen orthophosphate anhydrous, adjusted to pH 2.4 with orthophosphoric acid<br>C18 column (15 cm x 4.6 mm) | **HPLC**<br><br>**MP: 10 volumes of acetonitrile and 90 volumes of a solution containing 2.72% w/v of potassium dihydrogen phosphate, previously adjusted to pH 3.3 with orthophosphoric acid.**<br>**C18 (10 cm x 4.6 mm, 3.5 µ)**<br>**294 nm**<br>**2 mL/min** | HPLC<br><br>MP: ACN and buffer (3:22)<br>Buffer: 27.2 g/L of potassium dihydrogen orthophosphate anhydrous, adjusted to pH 3.3 with dil. phosphoric acid<br>C18 column (10 cm x 4.6 mm);<br>294 nm; 2 mL/min |

**Bold font assay methods are as updated in latest editions of IP, BP and USP (2022-2023)**

## 3.2 STABILITY PROFILES

A therapeutic agent which is not stable or is reactive may undergo degradation or structural change thereby altering its efficacy, and could produce degradants or derivatives that could compromise safety apart from providing misleading results during analysis by undergoing degradation or derivatization during testing process. Conditions of testing drugs exposes the molecule to routine conditions like hydrolysis, oxidation, high temperatures and light. Hence it is of paramount importance to study the chemistry of molecules and determine such conditions which trigger degradation. One of the ways to ascertain the stability of a molecule is the conduct of forced degradation study where severe conditions of temperature, humidity, and conditions of diverse pH are used in order to force the compound to degrade and produce degradants. With a thorough understanding of the chemistry of FQs and the specific conditions under which degradants are formed, measures could be evolved to avoid exposure to conditions not just during manufacture and storage but also during the testing of such drugs. During method development too, special measures are required to be enabled in case any particular condition has a damaging effect on the drugs under study, so that the method applied provides a true picture with regard to the quality of the compound analysed. It should be noted that certain experimental conditions applied during the testing produces degradants which in the natural course may not form and results of incorrect method of analysis could be misleading. To summarize, the analytical method should be specific and strong enough to detect drug and degradants if formed, but not be a cause for formation of degradants or derivatives due to application of extreme conditions adopted in the testing method.

### 3.2.1 HYDROLYTIC DEGRADATION

Hydrolysis is the cleavage of a chemical species by water. Forced drug degradation by exposure of drug solutions to acidic or basic conditions (or neutral) is useful to predict the primary hydrolytic drug degradation conditions and products[33].

Hydrolysis has a significant impact on the structural integrity of drugs in general. It has been reported that 71.6% of ethacrynic acid degraded after subjecting to stress degradation with 0.1 N HCl at 65 °C for 21 days, whereas 50% hydrolysis was seen in lidocaine with 6.5 N HCl at 108 °C in 25 h[8]. In case of azilsartan medoxomil potassium, acidic hydrolysis was carried out in 0.1 N HCl for 2 h at 60 °C, and 4 degradation products were noted[34].

While polythiazide was totally degraded in 1 h with 1N NaOH at 35 °C, norfloxacin subjected to degradative stress of 1 N NaOH at 100 °C for 15 h showed no degradation [7].

Researchers have studied the effect of strength of acid, duration and temperature on the acid hydrolytic degradation of CIP, and results of some of these studies have been tabulated below in Table 3.2 and graphically represented in the following Fig.3.1.

**Table 3.2 Acid Hydrolysis studies on CIP**

| Study No. | Normality of HCl (N) | Duration (h) | Temp (°C) | % of CIP Degraded | Ref |
|---|---|---|---|---|---|
| 1 | 0.1 | 4 | 25 | 60 | [35] |
| 2 | 0.1 | 4 | 80 | 0.6 | [36] |
| 3 | 0.5 | 5 | 50 | 2.0 | [37] |
| 3 | 1 | 4 | 80 | 13 | [36] |
| 4 | 5 | 22 | 40 | 91.3 | [38] |

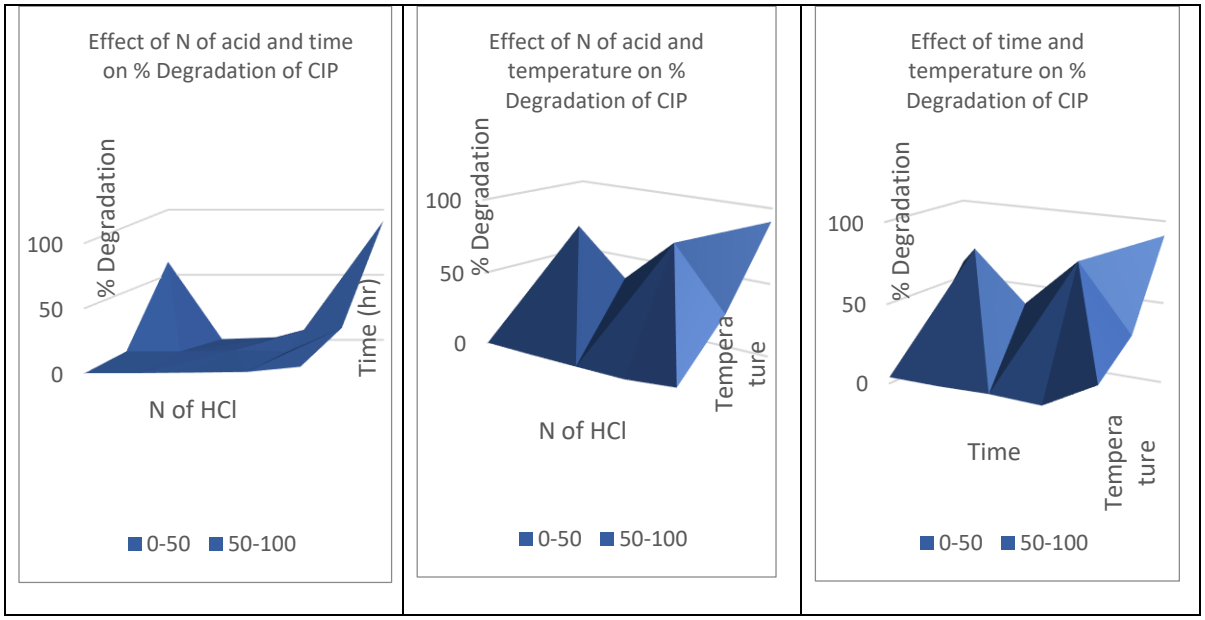


**Fig.3.1 Effect of strength of acid, duration and temperature on the acid hydrolytic degradation of CIP**

Similarly, effect of the variables on the acid hydrolytic degradation of LEV has been tabulated in Table 3.3 and graphically represented in the following Fig.3.2.

**Table 3.3 Acid Hydrolysis studies on LEV**

| Study No. | Normality of HCl (N) | Duration (h) | Temp (°C) | % of LEV Degraded | Ref |
|---|---|---|---|---|---|
| 1 | 0.5 | 168 | 70 | 3.3 | [39] |
| 2 | 0.1 | 12 | 25 | 4.8 | [40] |
| 3 | 5.0 | 6 | 60 | 34.4 | [41] |
| 4 | 0.1 | 1 | 80 | 8.12 | [42] |
| 5 | 0.1 | 2 | 60 | 0 | [43] |
| 6 | 1.0 | 3 | 25 | 14.5 | [44] |

During this study forced degradation of LEV and CIP was carried out using 5 N HCl, at 65 °C, for 6 h. No degradation was observed in case of LEV with NaOH.

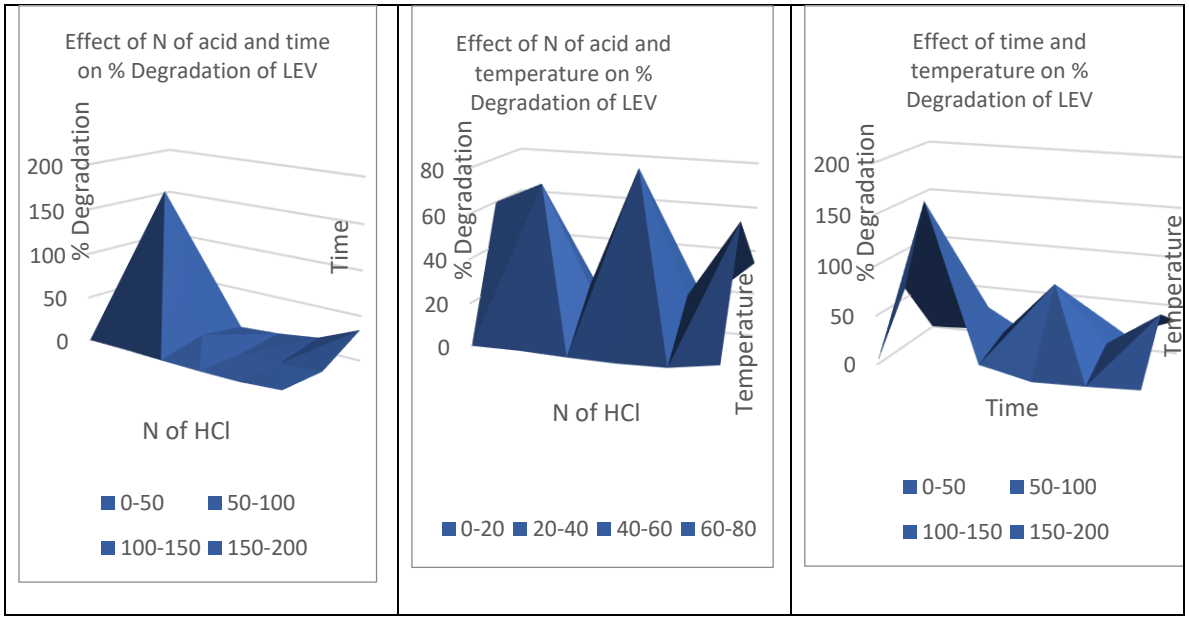


**Fig.3.2 Effect of strength of acid, duration and temperature on the acid hydrolytic degradation of LEV**

Stress degradation studies on levofloxacin showed significant degradation only upon acid hydrolysis, with descarboxyl levofloxacin as the major degradation product reported. Presence of other degradation products namely, desmethyl levofloxacin, levofloxacin-N-oxide and desethylene levofloxacin resulting from piperazinyl ring opening has also been reported along with other degradants. [45].

### 3.2.2 THERMAL DEGRADATION

Many APIs are sensitive to heat or tropical temperatures. When amlodipine was heated to 140 °C under atmospheric conditions and temperature maintained between 140 °C and 160 °C for 6 h, amlodipine was found to be fully degraded.

The FQs were exposed to a temperature of 60 °C for 6 h in a hot air oven and no degradation was observed.

The five FQs under investigation, namely, norfloxacin, ciprofloxacin, levofloxacin, ofloxacin and moxifloxacin, are reported to be stable to thermal degradation despite refluxing at 100 °C for 24 h[46]. This is in agreement with the reports of many other researchers[39,47].

### 3.2.3 OXIDATIVE DEGRADATION

Oxidation is one of the key reactions through which drugs undergo degradation in presence of molecular oxygen. Uncatalyzed oxidation of a substrate by molecular oxygen $O_2$ is termed as autoxidation. Autoxidation may start a chain process when the oxidized substrate, or superoxide, generates a reactive species that subsequently attacks additional substrate molecules in a sequence of steps[48].

``Initiation'' is either through autoxidation by molecular oxygen or by reaction of substrate with other endogenous chain-initiating radicals. Chain-initiating radicals may be generated through exposure of the system to light, heat, and catalytic levels of redox-active transition metals.

Ranitidine HCl suffers 37.8% loss in potency when exposed to 3% hydrogen peroxide at room temperature[7]. Significant oxidative degradation was also observed in case of LEV with 15% $H_2O_2$ in the dark, and levofloxacin-N-oxide was the major degradant[45].

The effect of strength of peroxide, duration and temperature on the oxidative degradation of CIP and LEV has been researched and results of some of these studies have been tabulated below in Tables 3.4 and 3.5 and graphically represented in the following Fig.3.3 and 3.4.

**Table 3.4 Oxidative degradation studies on CIP**

| Study | % of $H_2O_2$ | Duration (h) | Temp (°C) | Degradation | Ref |
|---|---|---|---|---|---|
| 1 | 3 | 24 | 25 | 1.1 | [37] |
| 2 | 3 | 4 | 80 | 3.5 | [36] |
| 3 | 6 | 22 | 40 | 91.8 | [38] |

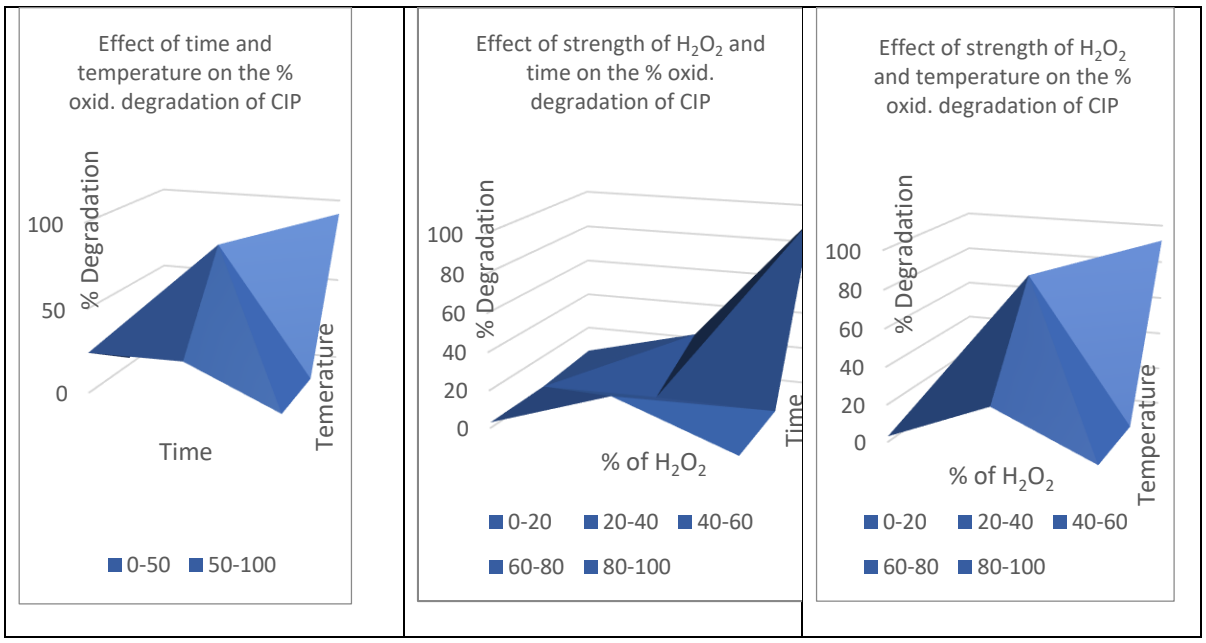


**Fig.3.3 Effect of strength of peroxide, duration and temperature on the oxidative degradation of CIP**

FQs are generally reported to be stable towards oxidation. However, forced oxidative degradation requires the presence of catalytic oxidizing agents like manganese dioxide ($MnO_2$) and chlorine dioxide ($ClO_2$) [49].

**Table 3.5 Oxidative degradation studies on LEV**

| Study | % of $H_2O_2$ | Duration (h) | Temp (°C) | Degradation | Ref |
|---|---|---|---|---|---|
| 1 | 0.01 | 12 | 25 | 12.7 | [39] |
| 2 | 30 | 0.08 | 25 | 12.3 | [40] |
| 3 | 30 | 1 | 25 | 9 | [41] |
| 4 | 3 | 1 | 80 | 66.44 | [42] |
| 5 | 3 | 0.5 | 60 | 3.9 | [43] |
| 6 | 3 | 3 | 25 | 13.2 | [44] |

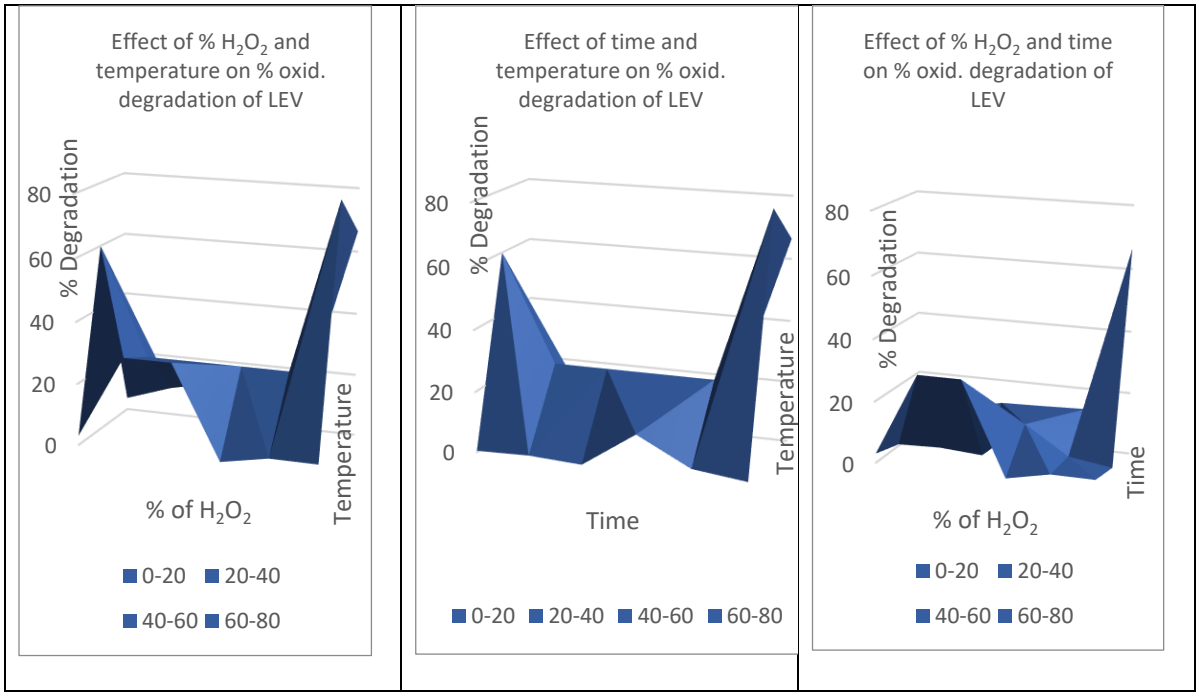


**Fig.3.4 Effect of strength of peroxide, duration and temperature on the oxidative degradation of LEV**

During the present study degradation was observed for LEV and CIP with 3% and 30% $H_2O_2$ at room temperature for 6 h.

The uneven slopes/ contours (Fig 3.1 to Fig 3.4) need to be construed as nonlinear behavior of drug exposed to extreme conditions or existence of certain other unknown parameters that determine the stability of the drug.

### 3.2.4 PHOTOLYTIC DEGRADATION

Photosensitive drugs are those molecules which undergo photodegradation i.e., degrade on exposure to light. From literature studied, it is evident that the fluoroquinolone class of therapeutic agents shows a tendency to undergo photolytic degradation.

The effects on photodegradation of sunlight, both natural and simulated, UV light at various wavelengths, irradiation power and duration of exposure have been observed by many researchers. Exposure may bring about an increase in temperature and this in turn will expedite degradation and may even change the reaction pathway leading to thermal degradation[50].

It is imperative that the molecule under investigation absorbs radiations at the wavelengths used for irradiation since degradation starts with absorbance of energy and molecules shifting to the excited state[51], and hence the source of light, its wavelength range, power of irradiation, temperature control provisions and many such factors play an important role in the study of photostability. An attempt to bring uniformity in such studies led to the introduction of Q1B guidelines by the ICH in 1996.

Drugs such as acetaminophen, amiodarone and dapsone, have been reported to get completely degraded in aqueous media on exposure to sunlight in 6 h[52]. Haloperidol underwent complete decomposition after 21 days exposure to daylight (transparent glass bottle)[53].

A detailed survey of literature revealed that conditions used for photodegradation studies were diverse and the results were not consistent, thus could not be presented graphically as has been done in case of acid hydrolytic and oxidative degradation of LEV and CIP through figures 3.1 to 3.4. The data has been reported in Tables 3.6 and 3.7.

## Table 3.6 Photodegradation studies on CIP

| Study | Wavelength λ (nm) | Power W | ICH guidelines | Solvent | pH | Duration (h) | Temp | Distance from source (cm) | Degradation % degraded | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | room light | | | | | 4 | | | 98.7 | [50] |
| 2 | room light | | | | | 96 | | | 91.2 | [50] |
| 3 | direct sunlight | | | | | 3months | | different packing | | [54] |
| 4 | 254 | | | | | 24 | 20 | 60 | 8.06 | [55] |
| 5 | 365 | | | | | 2712 | | | 15.56 | [56] |
| 6 | high pressure mercury lamp | | | 0.1M HCl | 1.2 | 4 | | | 60 | [57] |
| 7 | high pressure mercury lamp | | | citrate & borate buffers | 3 | 1 | | | 15 | [58] |
| 8 | high pressure mercury lamp | | | citrate & borate buffers | 8.6 | 1 | | | 85 | [58] |
| 9 | 254 | | | methanol | | 0.08 | | | 4.2 | [59] |
| 10 | 254 | | | methanol | | 0.25 | | | 5 | [59] |
| 11 | 254 | | | methanol | | 0.5 | | | 6.5 | [59] |
| 13 | medium pressure mercury lamp | 150 | | water, pH adjusted with NH4Cl or KCl | 9 | 2 min | | | 64 | [60] |
| 14 | medium pressure mercury lamp | 150 | | water, pH adjusted with NH4Cl or KCl | 9 | 4 min | | | 80 | [60] |

## Table 3.7 Photodegradation studies on LEV

| Study | Wavelength λ (nm) | Power W | ICH guidelines | Solvent | pH | Duration (h) | Temp | Distance from source (cm) | Degradation % degraded | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | sunlight | | | | | 48 | | | 1.6 | [40] |
| 2 | | | 1.2 m lux h | | | 72 | | | 1.6 | [40] |
| 3 | | | 1.2 m lux h | | | | | | 1.8 | [40] |
| 4 | Daylight | | | 0.9% NaCl | | 168 | | | 0 | [61] |
| 5 | Daylight | | | 5% dextrose | | 168 | | | 0 | [61] |
| 6 | Daylight | | | Ringer's | | 168 | | | 0 | [61] |
| 7 | Daylight | | | 0.9% NaCl | | 2016 | | | 4.89 | [61] |
| 8 | Daylight | | | 5% dextrose | | 2016 | | | 3.99 | [61] |
| 9 | Daylight | | | Ringer's | | 2016 | | | 14.4 | [61] |
| 10 | solar simulator | | | 0.9% NaCl | | 2016 | | | 5 | [61] |
| 11 | solar simulator | | | 5% dextrose | | 2016 | | | 8 | [61] |
| 12 | solar simulator | | | Ringer's | | 2016 | | | 12 | [61] |
| 13 | 280-400 | 0.119-0.125 $mW/m^2$ | | 0.1M HCl | | 2.5 | | 15.5 | | [62] |
| 14 | | 200 W h $m^2$ | 1.2 m lux h | | | 264 | | | 0.7 | [39] |
| 15 | Sunlight | | | | | 120 | | | 27.82 | [42] |
| 16 | | 200 W h / $m^2$ | | | | | | | 0 | [41] |
| 17 | 254 | | | | | 168 | | | 0.18 | [43] |
| 18 | Sunlight | | | | | 12 | | | 13.3 | [44] |

### 3.3 Process Related Impurities and Degradants.

The literature search was extended to include various related substances included in compendial monographs for the selected FQs. This helped to distinguish between process (synthesis) related impurities and degradants among the related substances notified in pharmacopoeia. The data collected has been reported in Table 3.8.

**Table 3.8 List of Impurities and degradants of selected Drugs**

| DRUG | PROCESS RELATED IMPURITIES | DEGRADANTS |
|---|---|---|
| CIP | Fluoroquinolonic acid[63,64] | Desethylene ciprofloxacin (metabolite and photodegradant too)[63,65–67] |
| | Desfluoro ciprofloxacin[63] | Decarboxy ciprofloxacin (through photodegradation or photo-induced oxidation) [60,63] |
| | 7-chloro 6-piperazine derivative[64,68] | 6-DesFluoro 6-Hydroxy Ciprofloxacin (photodegradant) [60,63] |
| LEV | Levofloxacin D-isomer (inactive isomer)[69,70] | Levofloxacin-N-oxide (inactive metabolite, photodegradant and oxidative degradant) [61,71–73] |
| | N-Desmethyl levofloxacin (active metabolite)[73–76] | Decarboxy Levofloxacin (formed through photodegradation or photo-induced oxidation) [73,77] |
| | 9-Desfluoro levofloxacin[76] | N-Desmethyl levofloxacin (photodegradant) [45,73] |
| | Decarboxy Levofloxacin[76] | N,N-Desethylene Levofloxacin (photodegradant) [45,78] |
| | Difluoro Levofloxacin[79–81] | |

| | | |
|---|---|---|
| | N,N-Desethylene Levofloxacin[78,82] | |
| MOX | 6,8-difluoro moxifloxacin [83,84] | Decarboxylated Moxifloxacin (acid hydrolytic degradant) [83–86] |
| | 6,8-dimethoxy moxifloxacin [83,84] | |
| | 8-ethoxy, 6-fluoro moxifloxacin [83,84] | |
| | 8-fluoro, 6-methoxy moxifloxacin [83,84] | |
| | 6-fluoro, 8-hydroxy moxifloxacin [72,84,87] | |
| | Ethyl 1-cyclopropyl-6,7-difluoro8-methoxy-4-oxo-1,4- dihydroquinoline-3-carboxylate[72] | |
| | 1-Cyclopropyl-6,7- difluoro-8-methoxy-4- oxo-1,4-dihydroquinoline3-carboxylic acid[72] | |
| | 1-Cyclopropyl-6,7-difluoro8-hydroxy-4-oxo-1,4- dihydroquinoline-3-carboxylic acid[72] | |
| | 1-Cyclopropyl-6-fluoro-8-methoxy-7-((4aS,7aS)-1-methyltetrahydro1H-pyrrolo[3,4-b] pyridin-6(2H,7H,7aH) - | |

| | | |
|---|---|---|
| | yl)-4-oxo-1,4-dihydroquinoline3-carboxylic acid[72] | |
| NOR | 7-chloro-6-fluoro-norfloxacin[88–91] | 1-ethyl-6-fluoro-7-(piperazin-1-yl) quinolin-4(1H)-one<br><br>Decarboxylated deriv (photodegradant and oxidative degradant)[92–96] |
| | 6,7-bis(piperazin-1-yl) norfloxacin[92] | 7-[(2-aminoethyl) amino] norfloxacin<br><br>Ethylenediamine derivative (photodegradant and oxidative degradant)[92–95,97] |
| | 7-chloro -6-(piperazin-1-yl) norfloxacin[92] | 6-fluoro-7-(4-formylpiperazin-1-yl) norfloxacin (photodegradant)[92,94,95] |
| | 6-chloro -7-(piperazin-1-yl) norfloxacin[92] | 7-amino derivative (photodegradant) [94,95] |
| OFL | N-Desmethyl ofloxacin[98,99] | Ofloxacin-N-oxide (photodegradant and oxidative degradant) [98,99] |
| | Desfluoro ofloxacin[98,99] | Decarboxy ofloxacin (photodegradant) [98,99] |
| | Decarboxy ofloxacin[98,99] | |
| | Difluoro ofloxacin<br><br>(difluoro pyrido benzoxazine carboxylic acid)[99] | |
| | 9-methyl Piperazino ofloxacin[98–100] | |

### 3.4 Review of Reported Methods

HPLC methods reported in literature were compiled and compared with proposed methods. The methods found in literature are presented in Table 3.9, 3.10, 3.11, 3.12 and 3.13 for CIP, LEV, MOX, NOR and OFL respectively.

**Drug 1: Ciprofloxacin**

A detailed literature search revealed 66 HPLC methods, among which 10 reported methods used gradient elution, 41 methods used fluorometric or MS detection, 9 methods needed specialized techniques or rare columns whereas 10 of the methods worked at elevated temperatures. The rest of the reported methods were found to be using a higher proportion of organic phase as compared to developed method.

## Table 3.9. HPLC methods reported in literature in case of Ciprofloxacin

| Sr. No. | HPLC Method variables | | | | | Retention time for drug peak (min) | Acid/ Base/ Neutral hydrolysis/ Oxidation/ Photolytic/ Thermal (Or stability) | Degradants/ Peak (Name and structure) | Comparison with Developed Method (DM) with Reported Method (RM) | Remarks | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Column | Column temp. (°C) | Mobile Phase | Flow rate (mL/min) | Detection (FL/ UV) (nm) | | | | | | |
| 1 | Polystyrene-divinylbenzene RP (PLRP-S) | 30 | 0.02M trichloroacetic acid (pH 3.0) :Acetonitrile: methanol (74:22:4) | 0.7 | FL | apprx. 8.0 min from chromatogram | Freeze -thaw | | Specialised column and FL detection for RM | Metabolites<br><br>Fluorometric detection | [101] |
| 2 | C18 | 35 | Gradient<br><br>Acetonitrile: Phosphate buffer (20 mM adjusted to pH 3 with PA; 16mL of Tetramethylammonium hydroxide to 1L buffer) | 1.5 | FL | 4.7 | No degradation after keeping 10hr in autosampler | No structures | Gradient and FL detection in case of RM | Metabolites in plasma | [102] |
| 3 | C18 | RT | Water: Acetonitrile:Triethylamine (80:19:1) | 1.4 | 275 | 4.6 | Not mentioned | No structures | | Residues in eggs after end of treatment | [103] |
| 4 | C18 | 50 | Acetonitrile:methanol:5%acetic acid (5:5:90) | 1.0 | 280 | Apprx 12min as mentioned | Not mentioned | No structures | Elevated temp for RM | IS lomefloxacin | [104] |
| 5 | Pentafluorophenylpropyl | | Gradient<br><br>Potassium hydrogen phosphate (0.05 | 1.0 | FL | 14.843 (from chromatogram) | -70°C, freeze-thaw, autosampler, | No structures | Gradient elution and FL detection for RM<br><br>Specialised column | IS NOR | [105] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | mol/L, pH 3 adjusted with phosphoric acid) and acetonitrile | | | | 5°C, daylight at RT | | | | |
| 6 | C18 | | Gradient<br><br>Acetonitrile: Potassium Phosphate buffer (5mM, pH3) | | FL | | Photodegradation in presence of org matter | | Gradient elution and FL detection for RM | Mentioned as modification of method 43 by Yorke | [106] |
| 7 | Column switching C18 to C8 | | Mobile phase 1 -20mM phosphate buffer pH 3.5 : Methanol (97:3) to Mobile phase 2- 20mM phosphate buffer pH 3.5: Acetonitrile (85:15) | 1.0 | FL | 9.58 | No | No | Specialised techniques and FL detection for RM | 2 columns | [107] |
| 8 | C18 | Ambient | 0.4M citric acid: methanol: Acetonitrile (10 :3:1) | 1.0 | FL | 7.52 | No | No | FL detection for RM | Pipemidic acid IS (Rt 4.88) | [108] |
| 9 | C18 | Ambient | Acetonitrile: 2% acetic acid (16:84) | 1.0 | 280 | 6.5 | RT 48hr<br><br>Freeze thaw cycles | No | | Umbelliferrone as IS (Rt 13.2) | [109] |
| 10 | C18 | | Acetonitrile: buffer (40:60)<br><br>Buffer- 2g sodium acetate, 2g sodium citrate, 1mL Triethylamine in 850mL water, pH 4.5) | 1.5 | FL | 1.06 | Stability in infusion bags containing saline & glucose | No | FL detection for RM | OFL & pefloxacin ( different method parameters for each) | [110] |
| 11 | C18 | 40 | Methanol: 25mM phosphate buffer pH 3.0 (28:72) | 1.0 | 293 | 3.42 | No | No | Elevated temp for RM | LEV (Rt 2.86) & MOX(Rt 9.98) | [111] |
| 12 | C18 | | Linear gradient | | 280 | | | | Gradient elution for RM | simultaneous determination of grepafloxacin, ciprofloxacin, and theophylline in human plasma and urine | [112] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | C18 | | Gradient<br>Methanol: Acetonitrile: formate buffer, 1M, pH3 | 1.0 | 280 | 3.19 | No | No | Gradient elution for RM | | [113] |
| 14 | C18 | RT | Water: Acetonitrile: Triethylamine (80:20:0.3); final pH 3.3 with Phosphoric acid | 1.0 | 279 | 2.45 | No | No | Higher proportion of ACN as compared to DM | CIP & NOR (Rt 2.3); not simultaneous | [114] |
| 15 | C18 | | Acetonitrile: methanol: acetate buffer (pH 3.6, 005M) (10:30:60) | 0.8 | FL | 5.1 | No | No | FL detection and high proportion of organic phase | Anthranilic acid as IS (Rt 8.8) | [115] |
| 16 | C18 | | 20:80 Acetonitrile : aqueous solution<br>Aq soln- 0.02 M potassium dihydrogen phosphate, 0.006 M PA, 0.012M tetraethyl ammoniumbromide, adjusted pH to 3 with 2M sodium hydroxide | 1.0 | FL | 2.28 | No | No | FL detection for RM<br><br>Multiple components in buffer of mobile phase for RM | Simultaneous with ENRO (Rt 3.3)<br><br>SARA as IS (Rt 4.4)<br><br>CIP as metabolite of ENRO | [116] |
| 17 | C18 | | 65% methanol in phosphate buffer<br><br>Buffer- (0.055 M potassium phosphate monobasic and 0.018M sodium phosphate dibasic) with 0.0055M hexadecyltrimethyl ammonium Bromide; adjusted to pH 7.4 with sodium hydroxide | 1.0 | 313 | 4.3 | No | No | High proportion of organic phase | Nalidixic acid as IS (Rt 7.3) | [117] |
| 18 | C8 | 50 | 0.16% *ortho*-phosphoric acid (adjusted to pH 3.0 with tetrabutyl ammonium hydroxide) with<br><br>20 ml/L acetonitrile per litre solution added after adjusted pH | 1.0 | FL | 6.0 | 16hr at 5°C | No | Elevated temp and FL detection in RM | ENR (Rt 10min) | [118] |
| 19 | C18 | Ambient | Acetonitrile: 0.025M PA adj.to pH 3 with Tetrabutylammonium hydroxide | 2.0 | FL | 4.0 | No | No | FL detection in RM | Comparison with microbio assay | [119] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 | C18 | Ambient (23° C) | 10mM Sodium dodecyl sulphate, 10 tetrabutyl ammonium acetate 25mM citric acid with 43% Acetonitrile | 1.0 | 280 (UV) & FL | 9.0 | No | No | FL detection in RM | LEV as IS (Rt 7.0min) | [120] |
| 21 | C18 | | Acetonitrile:PA: Tetrabutylammonium hydroxide (100: 1.67:15) in 1 L water | 2.0 | FL | 2.7 | No | No | FL detection in RM | IS (A-56619) Rt 4.0<br><br>NOR (Rt 2.3) | [121] |
| 22 | C18 | | 0.01M phosphate buffer pH 2.6 :Methanol (82:18) | 2.0 | 277 | 8.3 | Solution stability at RT | No | Higher proportion of organic phase (meth) coupled with high flow rate will give high back pressures | OFL as IS (Rt 10.6) | [122] |
| 23 | C18 | ambient | (0.04M) 1.9ml PA with 1.4g Tetrabutyl amm.iodide, 300 ml methanol, 700ml water; pH 2.2 | 1.0 | 278 | 7.5 | | | High proportion of organic phase | Serum<br><br>NOR(Rt7.0),OFL (Rt6.5) & peloxacin (Rt 6.5)<br><br>IS DL8357 (Rt 4.1) | [123] |
| 24 | C8 | | (41:59) 0.018M Phosphate buffer+ 0.1% Triethylamine, pH adj to 3 with PA :methanol | 1.5 | 270 | 3.522 | Stress testing:<br><br>5M NaOH & HCL, 40°C/ 75%RH for various time intervals<br><br>Oxid.6% $H_2O_2$ at 40°C/ 75%RH for 22hr | 6 peaks under acid, base & thermal<br><br>2 peaks under oxid & photolytic<br><br>(could be of CIP or dexa) | High proportion of organic phase | Formulations, serum, urine<br><br>Dexamethasone (Rt 7.628) | [38] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Thermal: at 40°C/ 75%RH for 22hr<br><br>Photolytic: sunlight for 1.25 hr | | | | |
| 25 | C18 | 40 | 0.1%PA, pH 3: methanol (70:30) | 0.6 | FL | 3.2 | Stress degradation:<br><br>N NaOH/ HCl, 30min, 60°C<br><br>3% $H_2O_2$, 30min, 60°C<br><br>UV chamber, 4 days<br><br>Thermal- 80°C, 4 hr | No | Elevated temp and FL detection in RM | | [124] |
| 26 | C18 for both methods | Ambient | A- Tetrahydrofuran: Acetonitrile: 1-hexanesulphonic<br><br>acid sodium (0.005 M, pH 3.0 with 0.1 M phosphoric<br><br>acid) (10:5:85, v/v/v)<br><br>B-<br><br>Tetrahydrofuran: Acetonitrile: 1 -hexanesulphonic | 1.0 | A: 254<br><br>B: 220 | 12 | Impurities used | | THF not healthy for humans and for PEEK material in HPLC system | 2 methods for 20 related compounds- 1 for CIP & components eluting before CIP (1st 12min). Other for components eluting after CIP (after 12min) | [125] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | acid sodium (0.005 M, pH 3.0 with 0.1 M phosphoric<br><br>acid) (25: 15:60, v/v/v). | | | | | | | Total run time 35min | |
| 27 | C8 | | Acetonitrile:methanol: citric acid (0.4mol/L) (7:15:78) | 1.2 | 275 | 8.566 | Solution stability 6months of refrigeration | No | 2 components in organic phase of mobile phase in RM | Serum & pharmaceuticals<br><br>Enoxacin (Rt 6.571)<br><br>NOR(Rt 7.812)<br><br>OFL (Rt 7.266)<br><br>HCT as IS (Rt 4.272) | [126] |
| 28 | C18 | 40 | Acetonitrile: phosphate buffer adjusted to pH 2.7 with PA (23:77) | 1.0 | 278 | 8.5 | Freeze-thaw, autosampler and long term stability for 4 weeks (in plasma) | No | Elevated temp in RM | Plasma | [127] |
| 29 | C18 | | Acetonitrile:water:Triethylamine (25:75:1), pH adjusted to 6 with PA | 1.0 | 300 | 2.7 | RT for 6 days | No | Higher proportion of organic phase as compared to DM | Simultaneous with:<br>OFL(Rt 3.5)<br>Tinidazole (Rt 4.5)<br>Ornidazole (Rt 5.8) | [128] |
| 30 | C8 | 50 | 5%acetic acid : methanol: Acetonitrile (90:5:5) | 0.5 | 280 | 9 | Left standing for 15hr | No | Elevated temp in RM | Plasma<br><br>Lomefloxacin as IS (Rt 11min) | [129] |
| 31 | C8 | | 1%Triethylamine, pH3.0 : Acetonitrile (14:86) | 1.0 | FL | 14.5 | RT for 24 hr | No | FL detection | Serum | [130] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | -20°C for 3 months<br><br>Freeze-thaw cycles | | | With pazufloxacin (Rt 11.3) and LEV (Rt 12.8) from fig | |
| 32 | C8 | | Buffer- 0.068% PA adjusted to pH 3 with Triethylamine<br><br>Acetonitrile: buffer (20:80) | 0.5 | FL | 9.5 | no | No | FL detection | CIP as metabolite of ENRO<br><br>In bovine & porcine muscle<br><br>ENRO (Rt 12min from fig) | [131] |
| 33 | C18 | 25 | (30:70) Acetonitrile: water with 0.1% glacial acetic acid adjusted to pH 2.8 with PA | 1.0 | 299 | Not mentioned | 0.5N NaOH/HCl, 50°C, 5hr<br>3%, 25°C, 24hr<br>80°, 5hr<br>Photo: white florescent lamp, 1.2 million lux hours and near ultraviolet (UV) 200 watt/m2/h at 25°C | Impurity (EDA) used | Higher proportion of organic component in mobile phase of RM | With ornidazole.<br><br>Impurity (EDA) used.<br><br>No data on Rt, no chromatogram available | [132] |
| 34 | Pentafluorophenyl core-shell | 25 | Gradient<br><br>Acetonitrile: phosphate buffer 20mM, pH 2 | 1.0 | UV DAD 280<br><br>& FL (different | 4.7 (from chromatogram) | 24hr at 4°C;<br><br>-80°C for 2months; | No | Specialised column<br><br>Gradient elution in RM | Urine & plasma<br><br>With LEV (Rt3.8), MOX (Rt 7.1) & gemi (Rt | [133] |

| | | | | | | | | | | | |
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| | | | | | ex& em for each drug) | | Freeze-thaw cycles | | | 7.3) from chromatogram | |
| 35 | C18 | Ambient | Acetonitrile: 0.1 M Sodium dihydrogen phosphate, pH 3.9(2:8) | 2.0 | FL | 4.55 | No | No | FL detection | Gingival crevicular fluid<br><br>IS Quinine sulphate (Rt13.25) | [134] |
| 36 | C18 | 30 | Gradient<br><br>Eluents-<br><br>Acetonitrile /Methanol/0.025M TBA·Cl/TFA<br><br>A : 75/25/899/1 B :150/50/799/1 both at pH 3.5 | 250µl/min | FL | 6.6 | Autosampler for 15hr<br><br>Freeze-thaw | No | FL detection and gradient elution in RM | Plasma<br><br>With MOX Rt 13), OFL (Rt 4.5) and IS (sarafloxacin Rt 10.6) | [135] |
| 37 | C18 | Ambient (23) | 10mM SDS, 10mM tert-Butyl acetoacetate, 10mM citric acid with 43% Acetonitrile adjusted to pH 3.5 | 1.0 | UV 293<br><br>FL | 5.5 (from chromatogram) | RT for 24hr<br><br>-70°C for 3months<br><br>Freeze-thaw cycles | No | High organic component in mobile phase of RM, multiple components in RM<br><br>FL detection | Plasma<br><br>LEV<br><br>Gati<br><br>MOX<br><br>Trovafloxacin<br><br>Cinoxacin<br><br>(Rt not clear) | [136] |
| 38 | C18 | 26 | Gradient<br><br>A: 0.4% phosphoric acid, pH 2.45 | 1.2 | 280 (for CIP) 293 | 9.5 | -20°C for 6 months<br><br>Freeze-thaw | No | Gradient elution | LEV(Rt 8.9)<br><br>MOX(Rt 14.9) | [137] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | B: methanol: Acetonitrile (1:3) | | | | | | | Pefloxacin(Rt 9.2)<br><br>Gatifloxacin (as IS) (Rt 12.1) | |
| 39 | C12 | 40 | 0.025M PA: methanol: Acetonitrile (75:13:12), pH adjusted to 3.0 with Triethylamine | 1.0 | FL | 3.6 | Stored at various temp 7 checked at 7 & 30 days | No | Elevated temp in RM<br><br>FL detection | Plasma<br><br>Sarafloxacin as IS (Rt 7.0) | [138] |
| 40 | UPLC BEH C18 | 45 | A: aq.0.1% formic acid-2mM ammonium acetate, pH 2.82<br><br>B: methanol. 0.1% formic acid-2mM ammonium acetate, pH 4.3 | 0.3 | MS | 1.02 | Freeze-thaw cycles<br><br>RT, 4°C, -20°C for 24hr to 3 months | No | Specialised technique<br><br>Elevated temp in RM<br><br>MS detection | Plasma<br><br>UPLC-MS but no MS data<br><br>LEV (Rt 0.86)<br>MOX (Rt 1.34)<br>Rifampicin (Rt 1.68) | [139] |
| 41 | C18 | 21 | 2.5%acetic acid: methanol: Acetonitrile (70:15:15) | 1.0 | 275 | 2.62min | No | No | High proportion of organic phase in mobile phase of RM | Ophthalmic solution | [140] |
| 42 | PLRP-S (polymeric) | 50 | 0.02M PA: Acetonitrile (85:15) | 0.8 | FL | 6.07-6.12 min | No | No | Elevated temp in RM<br><br>FL detection | 9 quinolones (simultaneous) in chicken tissue | [141] |
| 43 | C18 | | Acetonitrile: 0.25M PA (60:40) | 1.0 | 275 | 5.0 | No | No | High proportion of organic phase in mobile phase of RM | Serum, aqueous humour. Oph.drops, tablets.<br><br>Acetaminophen as IS (Rt 3.0) | [142] |
| 44 | C18 | Ambient | Acetonitrile: methanol: 1% acetic acid (8:8:84) | 1.5 | 280 | 7.7 | 0.1N NaOH, 0.1N | No | Both ACN and meth needed in mobile phase of RM | | [42] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | methanolic HCl for 24hr<br><br>254nm for 24hr<br><br>60°C for 24hr | | | | |
| 45 | C18 | | Acetonitrile:0.005 M tetrabutylammonium bromide (10:90) pH adjusted to 2.0 with phosphoric acid | 2.0 | 254 | 2.4 | | | | High flow rate.<br><br>Also pH 2.0 is not healthy for C!8 column | [143,144] |
| 46 | C18 | 25 | 0.02<br><br>*M* sodium phosphate buffer– Acetonitrile (65:35), pH 3, containing 0.2% triethylamine and 0.2% sodium dodecyl sulphate | 1.75 | 280 | 4.67 | Storage stability<br><br>Freeze thaw | No | With OFL & other FQs<br><br>Also identified LEV but not validate<br><br>Tried in different matrices | Higher proportion of Acetonitrile in RM | [145] |
| 47 | C18 | | Methanol: Phosphate buffer | | 277 & FL | | No | No | Comparison of method (C18 columns, ratio of meth & buffer) | More than 30% meth in all mobile phases | [146,147] |
| 48 | C18 | | Acetonitrile: tetrabutyl ammonium phosphate | | FL | 5.0 | No | No | Metabolites in body fluids | FL detection | [148] |
| 49 | C18 | | Phosphoric acid, Tetrabutylammonium hydroxide, Ceric ammonium nitrate | 2.0 | FL | 5.0 | no | No | Body fluids | FL detection | [149] |
| 50 | C18 | | pH 3 phosphate buffer/acetonitrile/<br><br>methanol (81/5/14) | 1.5 | FL | 8.03 | No | No | Serum, urine | | [150] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 51 | C18 | | ammonium phosphate pH 2.5, Acetonitrile, Methanol (80:13:7) | 4.0 | FL | 5.0 | No | No | Plasma | Higher proportion of organic phase<br><br>FL detection | [151] |
| 52 | C18 | ambient | methanol-acetonitrile-<br><br>0.4 M citric acid (3:1:10); | 2.0 | 275 | 9.5 | No | No | plasma<br><br>Pipemidic acid as IS | High proportion of organic phase | [152] |
| 53 | C18 | | Buffer containing phosphoric acid and Tetrabutylammonium hydroxide: Acetonitrile (88:12) | 2.0 | FL | 3.6 | No | No | Serum | FL detection | [153] |
| 54 | C18 | 40 | 0.1 ***M*** potassium phosphate, pH 2.5: Acetonitrile (81:19) | 0.8 | FL | 12.0 | No | No | Serum<br><br>With metabolites<br><br>Gradient for metabolites | FL detection | [154] |
| 55 | C18 | | 0.3% Tetrabutylammonium hydroxide in 0.02 *M* potassium dihydrogen phosphate, adjusted with phosphoric acid to pH 3.0: methanol: Acetonitrile (0.3: 14:5) | 1.7 | FL | 7.6 | | | Serum Urine<br><br>With metabolites | FL detection<br><br>Higher ratio of organic phase | [155] |
| 56 | C18 | | (3:1:10) Methanol: Acetonitrile: 0.4M citric acid | 1.0 | 275 | 10.51 | No | No | In body fluids | Higher ratio of organic phase | [156] |
| 57 | C18 | | 18 mM potassium dihydrogen phosphate with 0.13mM heptane sulphonic acid, methanol and concentrated phosphoric acid (700:300:1) | | UV (278) & FL | 8.5 | No | No | Serum<br><br>With NOR, OFL & other FQs<br><br>With CIP metabolites | Higher ratio of organic phase in RP<br><br>FL detection | [157] |
| 58 | Polystyrene divinyl | 30 | Acetonitrile: Methanol: 0.02M TCA adjusted to pH 3.0 (22:4:74) | 0.5 to 1.0 | UV (277) & FL | 9.5 (from chromatogram) | No | No | Serum, bile, saliva. | Specialized column | [158] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | benzene<br><br>C18 | | | | | | | | With CIP metabolites | | |
| 59 | C18 | RT | water: methanol: Acetonitrile (90:5:5) | 1.0 | FL | | | | With NOR & OFL<br><br>Blood, serum, urine | FL detection | [159] |
| 60 | C18 | | Acetonitrile-0.025 M orthophosphoric acid adjusted to pH 3.0 with Tetrabutylammonium hydroxide (11:89) | 1.5 | FL | 3.1 | No | No | Serum, urine<br><br>with NOR | FL detection | [160] |
| 61 | C18 | | Acetonitrile-0.025 M phosphoric acid adjusted to pH 3.0 with Tetrabutylammonium hydroxide (5:95) | 2.0 | FL | 3.5 | No | No | Plasma, serum, urine | FL detection | [147] |
| 62 | Immuno affinity chromatography (HPIAC) | | | | FL | | | | Uses antiFQS | HPIC column<br><br>FL detection | [161] |
| 63 | Phenyl | 30 | Gradient<br><br>1% formic acid adjusted to pH 3.0with ammonia : Acetonitrile | 0.5 | FL- &MS | | No | No | Multiresidues in egg | Specialised instrumentation/ columns/ detection<br><br>Gradient elution | [162] |
| 64 | Phenyl | 30 | Gradient<br><br>1% formic acid adjusted to pH 3.0with ammonia : Acetonitrile | 0.5 | FL- &MS | | No | No | Multiresidues in egg | Specialised instrumentation/ columns/ detection<br><br>Gradient elution | [163] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 65 | C18 | | Acetonitrile: phosphate buffer 0.02M | 1.0 | FL | 4.317 | No | No | With Sara, flume, enro, oxo.<br><br>In Salmon muscle tissue | FL detection | [164] |
| 66 | C18 | ambient | Acetonitrile: water with 0.04M PA, 0.1M Sodium dihydrogen phosphate, 0.5% sodium dodecyl sulphate and 0.005 M acetyl hydroxamic acid (42:58) | 1.0 | FL | | No | No | | Multicomponent aqueous phase, higher proportion of Acetonitrile, FL detection in RM | [165,166] |

**Drug 2: Levofloxacin**

A detailed literature search revealed 45 HPLC methods, among which 9 reported methods used gradient elution, 9 methods used fluorometric detection, 7 methods needed sophisticated techniques or rare columns whereas 9 of the methods worked at elevated temperatures. The rest of the reported methods were found to be using a higher proportion of organic phase as compared to developed method.

## Table 3.10. HPLC methods reported in literature in case of Levofloxacin

| Sr. No. | HPLC Method variables | | | | | Retention time for drug peak (min) | Acid/ Base/ Neutral hydrolysis/ Oxidation/ Photolytic/ Thermal (Or stability) | Degradants/ Peak (Name and structure) | Comparison with Developed Method (DM) with Reported Method (RM) | Remarks | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Column | Column temp. (°C) | Mobile Phase | Flow rate (mL/min) | Detection (FL/ UV) (nm) | | | | | | |
| 1 | C18 | Ambient (23) | 10mM SDS, 10mM TBAA, 10mM citric acid with 43% Acetonitrile adjusted to pH 3.5 | 1.0 | UV 293<br>FL | 5.5 (from chromatogram) | RT for 24hr<br>-70°C for 3months<br>Freeze-thaw cycles | No | DM has less components in buffer | Plasma<br>LEV<br>Gati<br>MOX<br>Trovafloxacin<br>Cinoxacin<br>(Rt not clear) | [167] |
| 2 | C18 | RT (25) | Acetonitrile: 0.4% Triethylamine, pH adjusted to 3.0 with Phosphoric Acid (24:76) | 1.0 | FL | 1.7 | Freeze-thaw cycles<br>3 months at -20°C | No | DM uses UV detector which is more popular | Plasma<br>MOX as IS (3.7) | [168] |
| 3 | C18 | RT (25) | Acetonitrile: 0.3 % Triethylamine adjusted to pH 3.3 with Phosphoric Acid (20:80) | 1.0 | 287/ 295 | | | | Stability indicating capacity of RM not mentioned<br>High org phase | | [169,170] |
| 4 | UPLC<br>BEH C18 | 30 | Acetonitrile: buffer (23: 77), (buffer: 20mM Dipotassium hydrogen phosphate + 0.1% Triethylamine, pH 2.5 by Ortho phosphoric acid). | 0.4 | 294 | 1.4 | 0.1M HCl/NaOH, 80°C for 1 hr<br>3% $H_2O_2$, 80°C 1hr<br>Thermal: 80°C 1hr | | DM uses usual C18 column | % degradation reported; highest in oxid.<br>No data of retention times | [42] |

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| | | | | | | | Sun 48 hr | | | | |
| 5 | C18 | 20 | Gradient | 0.25 | FL | 8.3 | 4°C , 14 days<br><br>RT, 12 hr<br><br>Autosampler, 15 hr | No | DM is isocratic<br><br>And uses UV detection | Plasma<br><br>IS MOX 16.7 | [171] |
| 6 | C18 | 25 | Gradient Acetonitrile:0.6% Triethylamine adjusted to pH 3 with Ortho Phosphoric acid | 1.2 | 284 | | Freeze-thaw, 37°C,<br><br>Long n short term | | | Biomimetic media | [172] |
| 7 | C18 | 25 | 0.05M citric acid monohydrate: 1 M ammonium acetate : Acetonitrile (84:1:15) | 1.0 | 293 | 11.2 | 0.1N HCl/NaOH and 3% $H_2O_2$, 60°C for 2hr, thermal 105°C for 48hr, uv for 7days | No | | In injection | [43] |
| 8 | C18 | 30 | 0.5% glacial acetic acid adjusted to pH 3.0 with ammonia solution : methanol (45:55) | 1.0 | 290 | 5.4 | 1N HCl, 1 N NaOH, 3hr RT<br><br>3% $H_2O_2$, 2hr, RT<br><br>Thermal- 80°C 3hr<br><br>Sunlight for 12hr | No peaks reported (though seen in figure) | DM uses lower proportion of organic phase | With cefixime | [44] |
| 9 | C18 | 40 | 0.1mol/L Dipotassium hydrogen phosphate/Acetonitrile/<br><br>Methanol / Trifluoroacetic acid in a ratio of 80/15/5/<br><br>0.3 | 0.8 | 294 | 2.6 | 1M HCl, NaOH, 3% H | Prominent peak of oxidant seen in chromatogram, not mentioned in text | DM uses simpler MP with less components. RM uses elevated temperature. | Comparison of different suspension vehicles | [173] |
| 10 | C18 | 40 | 0.04 M Ortho phosphoric acid buffer adjusted to pH 3 with Triethylamine: Acetonitrile (87:13) | 1.0 | 284 | Around 6.5 from | RT, fridge for 48hr | | RM uses elevated temperature. | Injection<br><br>With 5 hydroxy methyl furfural as degradant from | [174] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | chroma togram | | | | glucose/dextrose in formulation | |
| 11 | Zorbax SB-Phenyl | 45 | 0.094M phosphate buffer, methanol, Acetonitrile, Trifluoroacetic acid (80:5: 15:0.3) | 1.0 | 294 | Not mentioned<br><br>Around 15 min from chroma togram | Sunlight for 57days,<br><br>1N NaOH, $H_2SO_4$, 60°C for 2 hr | Known impurities and degradants used ( des-fluoro, des-methyl, diamine deriv, N-oxide) | RM uses elevated temperature, phenyl column, more components in MP | % degradation in acidic conditions mentioned | [175,176] |
| 12 | C8 | 40 | 80:20 ( water, pH adj with Ortho phosphoric acid: methanol) | 0.8 | 270 | 3.0 | | | RM uses elevated temperature, C8 column, no stability indicating data. | Combined tablet with cefpodoxime proxetil | [177] |
| 13 | C18 | 40 | 0.01 M Dipotassium hydrogen phosphate:methanol:0.5 M tetrabutyl ammonium hydrogen sulphate (75:25:4) | 1.0 | 290 | 4.84 | | | RM uses elevated temperature, no stability indicating data. | CIP as IS (6.74min) | [178] |
| 14 | C18 | 32 | 20mM Dipotassium hydrogen phosphate (pH 2.5): Acetonitrile (80:20) | 1.0 | 235 | 5.9 | | | RM uses controlled temperature, no stability indicating data. | Gatifloxacin as IS (10.1min) | [179] |
| 15 | C18 | 40 | Methanol: 25mM phosphate buffer pH3 (28:72) | 1.0 | 293 | | | | RM uses elevated temperature, no stability indicating data. | 3 FQs LEV, CIP, MOX<br><br>Rt not clear | [111] |
| 16 | C18 | ambient | Methanol: water (70:30) | 1.0 | 294 | 2.1 | | | RM reports no stability indicating data.<br><br>High meth ratio | | [180] |
| 17 | Column | | | | | | | | DM is simple, no column switching | | [181] |

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| | switching | | | | | | | | | | |
| 18 | C18 | 40 | 0.1%FA in methanol :0.1%FA in water (21:79) | 0.5 | | 1.9 | | | DM does not use elevated temp | CIP (2.5)<br><br>IS is Enro (2.8min) | [182] |
| 19<br><br>UPLC | WatersAcquity HSS T-3 C18 | 50 | Gradient<br><br>0.1% Triethylamine: Acetonitrile | 0.45 | 288 | 2.319 | 0.1 N NaOH/HCl<br><br>105°C, 96hr<br><br>30%$H_2O_2$<br><br>UV &sunlight 96hr | | DM is isocratic, simple HPLC (not UPLC), does not use elevated temp | UPLC | [183] |
| 20 | C18 | | Gradient<br><br>0.01M Sodium dihydrogen phosphate, pH2.7: Ceric ammonium nitrate | 1.5 | FL | 7.1 | | CIP (7.7min)<br><br>Desmethyl Lev (7.7min)<br><br>Lev N-oxide | DM uses isocratic mode, popular UV detection | With MOX 9.7min | [184] |
| 21 | C18 | 25 | Gradient<br><br>0.1% FA adjusted to pH 3.0 with Triethylamine: methanol: Acetonitrile | 1.0 | FL | 3.6 | | | DM uses isocratic mode, popular UV detection<br><br>RM does not provide stability data | With GAT, PAZU, MOX,<br><br>NOR as IS (4.4) | [185] |
| 22 | C8 | | 1%Triethylamine (pH3): Acetonitrile (86:14) | 1.0 | FL | 12.789 | RT, freeze thaw, -20°C | | DM uses popular C18 column and UV detection | CIP(14.512)<br><br>Pazu(11.248) | [130] |
| 23 | C8 & C18 | 40 | Gradient<br><br>15mM citrate buffer, pH adjusted to 3.2 with 5N sodium hydroxide: methanol: Acetonitrile | 1.5 | 280 | 7.8 from chromatogram | | | DM is isocratic, does not use elevated temp.<br><br>No stability data in RM | 7 FQs<br><br>Marbo as IS | [186] |

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| 24 | C18 | 30 | $PO_4$Buffer: Acetonitrile: methanol with Triethylamine to pH 5.2 (650:250:100) | 1.0 | 220 | 3.38 | | | RM uses controlled temperature, no stability indicating data. | With ambroxol (6.08) | [187] |
| 25 | C18 | | 1.5 mM potassium dihydrogen phosphate pH adjust to 4.5 with phosphoric acid) with0.0125% Triethylamine–methanol (70:30) | 1.0 | 292 | Not mentio ned | | | DM uses lower proportion of organic component. No stability data in RM | With ceftriaxone, metronidazole | [188] |
| 26 | C18 | 25 | 10 mM phosphate buffer at pH 3.0 (containing 0.01%<br><br>triethylamine)–acetonitrile (76:24, v/v) | 1.0 | FL | | | | DM uses popular UV detection, lower proportion of organic component. No stability data in RM | Terazosin as IS | [189] |
| 27 | Pent afluo roph enyl core shell | 25 | Gradient<br><br>Phosphate buffer: Acetonitrile | 1.0 | UV 280<br><br>& FL | | | | DM uses popular C18 column with isocratic elution. RM does not mention stability data | With CIP, MOX, Gemi | [133] |
| 28 | C18 | ambi ent | Phosphate buffer pH3: Acetonitrile (40:60) | 0.7 | 295 | 2.448 | | | DM uses lower proportion of organic component | | [190] |
| 29 | C18 | 26 | Gradient<br><br>0.4% Phosphoric acid and 1:3 mixt of methanol: Ceric ammonium nitrate | 1.2 | 293 | 8.9 | | | DM uses isocratic mode.<br><br>RM does not mention stability data | With Peflo(9.2), CIP (9.5)& MOX(14.9)<br><br>Gati (12.1)as IS | [137] |
| 30 | C18 | 35 | phosphate buffer (pH 2.5)–Acetonitrile (80:20) | 1.0 | 293 | 6.0 | | | DM does not use elevated temperature. No stability data in RM | LEV as IS<br><br>Gati(10.8) , Spar(12.8) & MOX(17.0) | [191] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 31 | UPLC BEH C18 | | Gradient<br><br>water-formic acid (100:0.1, v/v)-ammonium acetate 2 mM (A) and<br><br>methanol-formic acid (100:0.1, v/v)-ammonium acetate 2 mM | 0.3 | MS | 0.86 | | | RM comprises UPLC with MS detection and gradient mode.<br><br>DM is simple | LEV, MOX(1.34), CIP(1.02) and rifampicin(1.69) | [139] |
| 32 | C18 | 30 | 0.1%FA: O.1% FA in Acetonitrile (60:40) | 0.5 | MS | 2.58 | | | DM uses simple UV detection.<br><br>RM uses MS detection, higher organic component, elevated/ controlled temperature | Enro is used as IS (2.66)<br><br>With MOX(2.82) | [192] |
| 33 | C18 | | Acetonitrile: potassium dihydogen phosphate: methanol (20:70:10) | 1.2 | 306 | 3.433 | | | RM has more components in MP (both meth and Acetonitrile); no stability data | With Ambroxol (6.887)<br><br>QbD | [193] |
| 34 | C18 | | Methanol:0.01M ammonium acetate(70:30) | 1.0 | 265 | 3.0 | | | No stability data.<br><br>Chromatograms show peaks with less RS, shoulder for IS peak | With amoxicillin & lansoprazole.<br><br>Paracetamol as IS | [194] |

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| 35 | C18 | 25 | Acetonitrile:water (5:6) with Phosphoric Acid added to adjust pH to 2.9 | 1.0 | 260<br><br>265<br><br>270<br><br>275<br><br>280 | 3.8 | | | RM has high proportion of Acetonitrile, multiple wavelengths for multivariate analysis | With propylparaben as IS (7.8min) | [195] |
| 36 | C18 | 35 | Sodium dihydrogen ortho-phosphate buffer pH 2.5: Acetonitrile (80:20) | 1.0 | 293 | | | | RM reports low recovery for IS LEV (63.47%) | Gati, spar, moxi.<br><br>Levo as IS | [191] |
| 37 | C18 | | Acetonitrile: 10 mM *o*-phosphoric acid (25:75) | | FL | | | | More data not available | Moxi as IS | [196] |
| 38 | C18 | | Gradient<br><br>Acetonitrile:0.1 M phosphoric acid: sodium hydroxide buffer (pH 3.0):0.01 M n-octylamine (pH 3.0) | 1.0 | 292-LEV<br><br>294-MOX | 7.5 | No degradation studies | | Complicated buffer<br><br>Cefepime with 3 FQs, LEV, MOX and garenoxacin<br><br>Urine | MOX 8.9<br><br>Gare 10.5 | [197] |
| 39 | C18 | RT (24) | water: acetonitrile:phosphoric acid 0.025 M, pH adjusted to 3.0 with triethylamine (60:20:20) | 1.0 | 294 | 3.52 | No degradation studies | | Higher proportion of Acetonitrile<br>Compared with uv spectro mtd | | [198] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 40 | C18 | | Acetonitrile: water (80:20 v/v) adjusted to pH 3.5 by Ortho phosphoric acid | 1.4 | 296 | 1.38 | No degradation studies | | Plasma, saliva<br><br>Run time 2min | | [199] |
| 41 | C18 | | Acetonitrile:0.2%Triethylamine adjusted to pH 3.15 with Ortho phosphoric acid | 1.0 | 315 | 2.99 | No degradation studies | | Tailing of peaks 1.5 | Ornidazole 4.81 | [200] |
| 42 | C18 | 35 | Copper(II) sulfate pentahydrate (5 mM) containing L-isoleucine (10 mM)-methanol (87.5:12.5) | 1.0 | 330 | Levo 8min | | | Stereospecific<br><br>Chiral reagents added for chiral separation | D-isomer 10 min<br><br>CIP (IS) 13min | [201] |
| 43 | C18 | | Acetonitrile: methanol: phosphate buffer (pH 3) (17:3:80) | 1.0 | 295 | 7.66 | | | | CIP as IS (8.5min) | [202] |
| 44 | C18 | ambient | 25 mM sodium phosphate monobasic monohydrate and 0.1% trifluoroacetic acid (pH 2.4): Acetonitrile (86: 14) | 1.5 | 266 | 8.0 | No degradation studies | | plasma | CIP as IS (9.4 min) | [203] |
| 45 | C18 (18e) | | methanol: 0.025M potassium dihydrogen phosphate adjusted to pH 3 using Orthophosphoric acid (20:80) | 4.0 | 290 | 1.1 | No degradation studies | | Separation achieved in 5min. Requires special column | With lome (2), spar(2.98) and gati(4.3) | [204] |

**Drug 3: Moxifloxacin**

A detailed literature search revealed 54 HPLC methods, among which 15 reported methods used gradient or stepwise elution, 16 methods used fluorometric detection, 5 used MS detection, one used ECD detection,5 methods needed sophisticated techniques or rare columns whereas 7 of the methods worked at elevated temperatures. The rest of the reported methods were found to be using a higher proportion of organic phase as compared to developed method.

**Table 3.11. HPLC methods reported in literature in case of Moxifloxacin**

| Sr. No. | HPLC Method variables | | | | | Retention time for drug peak (min) | Acid/ Base/ Neutral hydrolysis/ Oxidation/ Photolytic/ Thermal (Or stability) | Degradants/ Peak (Name and structure) | Comparison with Developed Method (DM) with Reported Method (RM) | Remarks | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Column | Column temp. (°C) | Mobile Phase | Flow rate (mL/min) | Detection (FL/ UV) (nm) | | | | | | |
| 1 | C18 | 25 | Phosphate Buffer: Methanol (55:45) | 1.0 | 293 | 5.855 | No | No | Higher proportion of organic phase in RM | OPA adjusted with TEA (pH 6.0 or 2.5 not clear) | [205] |
| 2 | Column switching | 20 | 2 mobile phases | 1.0 & 1.2 | FL | 13.4 | No | No | Column switching and FL detection | With LEV and gatifloxacin | [181] |
| 3 | Column switching | 20 | 2 mobile phases | 1.0 & 1.25 | FL | 5.7 | No | No | Column switching and FL detection | | [206] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 | C18 | 50 | Gradient<br><br>0.01 mol/l tetrabutyl ammonium sulphate and 0.05 mol/l sodium dihydrogenephosphate (a, pH 3.0)<br><br>And Ceric ammonium nitrate | 1.1 & 1.3 | FL | 12.8 | No | No | RM requires elevated temp, gradient elution and fluorimetric detection | HPLC compared with bioassay.<br><br>Biofluids | [207–210] |
| 5 | C8 | 50 | 0.16% Ortho phosphoric acid adjusted to pH 6 with Tetrabutylammonium hydroxide: Acetonitrile (100:5) | 1.5 | FL | 6.0 | no | No | RM uses elevated temp and fluorimetric detection | Serum sample | [211] |
| 6 | C18 | | Methanol: Acetonitrile: water (85:5:10) water pH adj to 2.75 | 1.0 | 290 | 2.0 | no | no | Higher proportion of organic phase in mobile phase of RM | Serum<br><br>Dead time needs to be checked | [212] |
| 7 | C18<br><br>Preparative | 45 | Gradient<br><br>A: 10 ml Phosphoric acid + 1 ml trifluoroacetic acid in 1000 ml water (pH adjusted to 2.2 with triethylamine) and methanol in the ratio of 85:15.<br><br>B: methanol and water in the ratio of 80:20 | 1.0 | 295 | | no | no | RM uses elevated temp and fluorimetric detection | With synthesis related impurities | [87] |
| 8 | C18 | 45 | Water with 2% Triethylamine adjusted to pH 6 : Acetonitrile (90:10) | 1.5 | 290 | 5.0 | no | no | RM uses elevated temp | With impurities | [213] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | C18 | | Gradient<br><br>Acetonitrile/0.1 M phosphoric acid/ sodium hydroxide buffer (pH 3.0)/0.01 M n-octylamine (pH 3.0) | 1.0 | 294 | 8.9 | no | no | RM uses gradient elution | With cefepime and LEV & garenoxacin.<br><br>Different wavelengths for each drug.<br><br>Urine sample. | [197] |
| 10 | C18 | Ambient (23) | 10mM SDS, 10mM tert-Butyl acetoacetate, 10mM citric acid with Acetonitrile adjusted to pH 3.5 (57:43) | 1.0 | UV 293<br><br>FL | 5.5 (from chromatogram) | RT for 24hr<br><br>-70°C for 3months<br><br>Freeze-thaw cycles | No | DM has less components in buffer | Plasma<br><br>LEV<br><br>Gati<br><br>MOX<br><br>Trovafloxacin<br><br>Cinoxacin<br><br>(Rt not clear) | [136] |
| 11 | LC-ESI/MS-MS<br><br>C18 | | 0.1% formic acid: Acetonitrile (60:40) | | MS | 2.75 | No | No | LC-ESI/MS-MS<br><br>Higher proportion of Acetonitrile in RM | In plasma<br><br>With lomefloxacin as IS | [214] |
| 12 | C18 | | Acetonitrile:10mM potassium dihydrogen phosphate buffer adjusted to pH 4 (18:82) | 1.25 | 296 | 6.0 | No | No | Higher proportion of Acetonitrile in RM | Plasma and lung samples | [215] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | C18 | 25 | Acetonitrile: 0.25 mol/L Trisodium phosphate pH 3 (5:95) | 1.0 | FL | 3.55 | No | No | FL detection | OFL as IS<br><br>Plasma | [216] |
| 14 | C18 | | Acetonitrile:10 mM orthophosphoric acid (pH 2.5) (80:20) | 1.2 | FL | 4.795 | No | No | FL detection<br><br>Higher proportion of Acetonitrile in RM | NOR as IS<br><br>Plasma | [217] |
| 15 | C18 | 35 | Gradient<br><br>Dihydrogen phosphate buffer 50mM :Acetonitrile | 1.0 | 270 & 280 (Changed during run at 14min) | 12.55 | Freeze-thaw | No | Gradient elution<br><br>Change in wavelength during run | Plasma<br><br>With LEV, linezolid, rifampicin.<br><br>Quinoxaline as IS | [218] |
| 16 | C18 | | Gradient<br><br>Acetonitrile: buffer<br><br>Acetonitrile from 20 to 41% | 1.0 | FL | 7.3 | No | No | Gradient elution<br><br>Higher proportion of Acetonitrile in RM | Plasma<br><br>Full details of HPLC method not provided but reference made to[210] | [219] |
| 17 | C18 | | Acetonitrile: 10mM potassium dihydrogen phosphate (19:81) | 1.0 | 296 | 8.82 | | | Higher proportion of Acetonitrile in RM | Serum<br><br>Full data not available | [220] |
| 18 | C18 | ambient | 50 mM phosphate buffer, pH 2.6 (adjusted with 1 N HCl) and Acetonitrile (80:20) | 1.5 | FL | 4.0 | No | No | FL detection<br><br>Higher proportion of Acetonitrile in RM | Human saliva<br><br>Plasma<br><br>OFL as IS | [221,222] |
| 19 | C8 | 25 | 18mm phosphate buffer with 0.1%Triethylamine adjusted to pH 2.8 with dilute phosphoric acid and methanol (38:62) | 1.5 | 254 | 3.449 | Acid, base (5M HCl and NaOH) for varied time. Thermal at 105°C | | High proportion of org phase in mobile phase of RM | With prednisolone | [223] |

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| | | | | | | | Oxidative deg with 6% $H_2O_2$<br><br>Photodeg under sunlight | | | | |
| 20 | C18 | ambient | 10mM sodium phosphate buffer: methanol (60:40) pH 4 | 1.0 | 294 | 7.8 | Forced degradation<br><br>Photodeg under daylight | Some peaks seen. No structure | High proportion of org phase in mobile phase of RM | | [224] |
| 21 | C18 | 25 | Methanol: water: Triethylamine (112.5:208.8: 0.06) | 1.0 | 289 | | | | High proportion of org phase in mobile phase of RM | Chinese journal.<br><br>Full data not available. | [225] |
| 22 | C18 | 40 | Methanol: 25mM phosphate buffer pH 3 (28:72) | 1.0 | 293 | | No | No | High proportion of org phase in mobile phase of RM<br><br>Elevated temp required for RM | Comparison of behavior of MOX, CIP and MOX on different columns. | [111] |
| 23 | C18 | | Gradient<br><br>Acetonitrile: 0.1mol/L sodium dihydrogen phosphate at pH 2.7 | 1.5 | FL | 9.7 | No | No | Gradient elution<br><br>FL detection | Plasma<br><br>With LEV | [184] |
| 24 | C18 | 35 | phosphate buffer (pH 2.5) and acetonitrile (80:20) | 1.0 | 293 | 16.0 | No | No | High proportion of organic phase in mobile phase of RM<br><br>Long retention / run time<br><br>RM requires column temp maintenance | Plasma<br><br>With gatifloxacin and sparfloxacin LEV as IS | [191] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | C8 | 30 | Acetonitrile: methanol:KH2PO4 buffer solution (0.02 M, containing 1% triethylamine, pH 3.0 adjusted with concentrated phosphoric acid) (15:20:65) | 1.0 | 296 | 7.6 | Freeze-thaw | No | High proportion of organic phase in mobile phase of RM<br><br>RM requires column temp maintenance | Plasma<br><br>Gatifloxacin as IS | [226] |
| 26 | C18 | 30 | Gradient<br><br>Acetonitrile/Methanol/ 0.025 M TBA·Cl/ Trifluoroacetic acid (pH 3.5 | 0.25 | FL<br><br>Different em & ex wavelengths for each drug | 13.0 | Freeze-thaw | No | Gradient elution<br><br>Fluorimetric detection in RM | Plasma<br><br>With CIP & OFL<br><br>Sarafloxacin as IS | [135] |
| 27 | C18 Waters Acc Q tag (amino acid analysis) | 30 | Gradient<br><br>Methanol: Acetonitrile: TBA.Cl : Trifluoroacetic acid | 1.0 | FL | 16.7 | No | No | Gradient elution,<br><br>Fluorimetric detection and special column in RM<br><br>Run/ Retention time too long | Aqueous & vitreous humor<br><br>With OFL<br><br>Sarafloxacin as IS | [227] |
| 28 | LC-ESI/MS-MS<br><br>C18 | 20 | Gradient<br><br>Water: Acetonitrile: buffer containing ammonium acetate, acetic acid and Trifluoroacetic acid | 0.3 | MS | 1.58 | Freeze -thaw, room temp, refrigerator | | Gradient elution,<br><br>MS detection in RM<br><br>Temp control needed | Plasma, CSF | [228] |
| 29 | C8 | | eco-friendly isocratic eluent; ethanol: water containing | 1.0 | 240 and 280 ( time based | 4.8 | No | No | High proportion of ethanol | Green method<br><br>In combination with other drugs | [229] |

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| | | | 0.05% triethanolamine (90:10, pH 4.5) | | program ming) | | | | Programming/ change of wavelength needed | dexamethasone, prednisolone | |
| 30 | C18 | RT | Acetonitrile: 0.4% Triethylamine, pH 3.0 (24:76) | 1.0 | 295 | 4.0 | Freeze-thaw | No | High proportion of organic phase in mobile phase of RM | LEV as IS | [230] |
| 31 | C18 | | methanol: potassium dihydrogen phosphate buffer (pH 3.2;<br><br>25 mM, 0.5% Triethylamine) (60:40) | 1.2 | 308 | 3.3 | No | No | High proportion of organic phase in mobile phase of RM | With ketorolac | [231] |
| 32 | dC18<br><br>(used for polar com poun ds) | | Gradient<br><br>Acetonitrile: 0.1% Trifluoroacetic acid | 1.0 | FL for MOX | 10.78 | No | No | Gradient elution,<br><br>Fluorimetric detection | Different UV wavelengths n FL detection for each drug<br><br>With CIP and sparfloxacin and other substances | [232] |
| 33 | LC-MS/ MS<br><br>(ESI-APC I com bine d mod e) | 30 | Methanol :0.03% Triethylamine (85:15) | 0.5 | MS | 2.1 | Freeze-thaw cycles | No | MS detection | With pyrazinamide and a prodrug.<br><br>Metronidazole used as IS | [233] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 34 | LC-MS/MS<br><br>C18 | | Gradient<br><br>Water: Acetonitrile: acetate buffer | 0.3 | MS | | No | No | Gradient elution and MS detection in RM | With cyanoimipramin as IS | [234] |
| 35 | C18 | | 0.01M phosphate buffer: Acetonitrile (50:50) | 1.0 | 254 | 2.58 | No | No | High proportion of organic phase in mobile phase of RM | With dexamethasone | [235] |
| 36 | C18 | ambient | Ammonium dihydrogen orthophosphate solution (pH adjusted to 3) and Acetonitrile (75: 25) | 1.5 | 295 | 4.0 | No | No | High proportion of organic phase in mobile phase of RM | | [236] |
| 37 | C18 | 25 | A-Phosphoric acid and tetramethyl amm hydroxide in water: Acetonitrile (95:5)<br><br>Acetonitrile: methanol (55:45), adjusted to pH 3 | 1.0 | 294 | 5.6 | | | High proportion of organic phase in mobile phase of RM | | [237] |
| 38 | C8 & C18 | 40 | Gradient<br><br>15mM citrate buffer, pH adj to 3.2 with 5N sodium hydroxide: methanol: Ceric ammonium nitrate | 1.5 | 280 | 7.8 from chromatogram | No | No | RM uses elevated temp. | 7 FQs<br><br>Marbo as IS | [186] |
| 39 | Pentafluorophenyl core shell | 25 | Gradient<br><br>Phosphate buffer: Acetonitrile | 1.0 | UV 280<br><br>& FL | | No | No | RM requires special column and gradient elution | With CIP, MOX, Gemi | [133] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 40 | LC/ESI-MS/MS | 30 | Step elution<br><br>10 mM ammonium formate in water-formic acid (99.9:0.1) and methanol | 0.5 | MS | 2.15 | RT<br><br>Refrigerator<br><br>Freeze-thaw | No | RM requires specialised detection and step elution | With CIP and some other drugs | [238] |
| 41 | C18 | | methanol: water: Acetonitrile, 60:45:5, pH 2.7 | 1.0 | Different wavelengths for each drug | 6.7 | No | No | High proportion of organic phase in mobile phase of RM | With cimetidine, famotidine and ranitidine. | [239] |
| 42 | C18 | ambient | Acetonitrile: Buffer pH 4 (60:40)<br><br>Ammonium acetate buffer adjusted to pH 4 with phosphoric acid | 1.0 | 294 | 3.54 | No | No | High proportion of organic phase in mobile phase of RM | With ketorolac | [240] |
| 43 | C18 | RT | 0.1%Triethylamine adjusted to pH 4.8 with phosphoric acid: Acetonitrile (80:20) | 1.0 | 296 | Beyond 15min | No | No | Retention time of MOX is beyond 15 min (longer run time)<br><br>High proportion of organic phase in mobile phase of RM | Plasma<br><br>CIP as IS | [241] |
| 44 | C18 | | 50 mM potassium dihydrogen phosphate (pH 2.4): Acetonitrile (77:23) | 1.5 | FL | 6.5 | Freeze-thaw<br><br>RT<br><br>daylight | No | FL detection<br><br>High proportion of organic phase in mobile phase of RM | Plasma<br><br>LEV as IS | [242] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 45 | C18 | ambient | Gradient<br><br>methanol and triethylamine phosphate buffer (pH 2.5) | 1.2 | 296 | 7.0 | No | No | Gradient elution in RM | With bedaquiline and pyrazinamide (different wavelengths for each) | [243] |
| 46 | C18 | | Acetonitrile : 0.01M Potassium dihydrogen phosphate (40:60) | 1.0 | 276 | 7.007 | No | No | High proportion of organic phase in mobile phase of RM | With cefixime | [244] |
| 47 | C18 | | Methanol, water and acetonitrile (45:30:25) at pH 4 | 1.0 | 295 | 2.35 | Accelerated stability studies | No | High proportion of organic phase in mobile phase of RM | In tablets, plasma<br><br>Benzoic acid as IS | [245] |
| 48 | C18 | | Methanol: water: triethylamine (60:40:0.75; pH adjusted to 3.25 with orthophosphoric acid | 0.8 | 244.2 | 4.23 | No | No | High proportion of organic phase in mobile phase of RM | With dexamethasone | [246] |
| 49 | C18 | 35 | Britton Robinson buffer pH 5.0 and methanol (93 : 7} | 0.5 | ECD | 5.075 | No | No | ECD detector | | [247] |
| 50 | C18 | 35 | 0.05 M monobasic phosphate buffer: acetonitrile (65:35 ) pH 6.0 | 1.0 | 265 | 5.7 | | | High proportion of organic phase in mobile phase of RM | Plasma, aqueous humor, formulations<br><br>With timolol maleate, dexamethasone and diclofenac sodium | [248] |
| 51 | C8 | | Methanol: Water (75:25 *v/v)* pH adjusted to 3.0 with ortho-phosphoric acid | 1.0 | 240 | 2.22 | No | No | High proportion of organic phase in mobile phase of RM | With dexamethasone in ophthalmic solution | [249] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 52 | C18 | | | | FL | | | | FL detection | Pharmacokinetic study<br><br>OFL as IS<br><br>Not much info available | [250] |
| 53 | C18 | | Methanol: Acetonitrile: ammonia buffer (20:20:60) | 1.0 | 290 | 6.2 | | | High proportion of organic phase in mobile phase of RM | Also for LEV | [251] |
| 54 | C18 | ambient | 0.1 M formic acid (pH 2.80) and methanol (30:70) | 1.0 | 280 | 2.0 | No | NO | High proportion of organic phase in mobile phase of RM | Rosuvastatin as IS | [252] |
| 55 | C18 | 45 | Methanol: 0.05% trifluoroacetic acid (38:62) | 1.1 | 290 | 8.0 | No | No | with OFL (3.8) | Higher proportion of methanol | [253] |

**Drug 4: Norfloxacin**

A detailed literature search revealed 36 HPLC methods, among which 3 reported methods used gradient or stepwise elution, 13 methods used fluorimetric detection, and 2 methods needed sophisticated techniques or rare columns whereas 6 of the methods worked at elevated temperatures. The rest of the reported methods were found to be using a higher proportion of organic phase as compared to developed method.

## Table 3.12. HPLC methods reported in literature in case of Norfloxacin

| Sr. No. | HPLC Method variables | | | | | Retention time for drug peak (min) | Acid/ Base/ Neutral hydrolysis/ Oxidation/ Photolytic/ Thermal (Or stability) | Degradants/ Peak (Name and structure) | Comparison with Developed Method (DM) with Reported Method (RM) | Remarks | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Column | Column temp. (°C) | Mobile Phase | Flow rate (mL/min) | Detection (FL/ UV) (nm) | | | | | | |
| 1 | C18 | 40 | 20mM sodium hydrogen phosphate buffer pH 3.0: Acetonitrile (80:12) | 1.2 | 280 | 10.5 | RT and freeze-thaw cycles | No | RM has elevated temp and longer run time | Plasma<br>CIP as IS | [254] |
| 2 | C18 | | 96-97% buffer<br>3-4% Tetrahydrofuran<br>Buffer: 30mM/L dipotassium hydrogen phosphate adjusted to pH 3 using HCl | | FL | | Photo induced degradation | | RM uses FL detection | Plasma, urine<br>With CIP, OFL, enoxacin<br>Irradiated and photodegraded | [255] |
| 3 | C18 | | 50mM Sodium dihydrogen phosphate<br>buffer: Acetonitrile: Methanol, (pH 2.5 adjusted orthophosphoric acid) 15:70:15 | 1.0 | 294 | 5.48 | No | No | RM uses very high proportion of organic phase | With ornidazole | [256] |
| 4 | C18 | | 5% acetic acid: methanol (80:20) | 1.0 | 277 | 5.7 | Heating, oxid, acid-base hydrolysis stress | No | Higher proportion of organic phase in RM | | [257] |
| 5 | C18 | RT (24) | Water: acetonitrile: triethylamine (80:20:0.3) pH 3.3 | 1.0 | 279 | 2.3 | No | No | Higher proportion of Acetonitrile as | With CIP | [114] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | compared to DM | | |
| 6 | C8 | ambient | Sodium phosphate buffer pH 3.0 (85:15) | 2.0 | 278 | 6.29 | Photodeg (sunlight, uv, fluorescent) | 4.23 | Higher flow rate in RM (more consumption of Acetonitrile) | | [97] |
| 7 | C5 | 30 | Gradient<br><br>Acetonitrile: Tetrahydrofuran: potassium phosphate buffer pH 2.6 | 1.0 | UV and FL | 9.91 | No | No | Gradient elution and FL detection in RM | Auto extraction from animal feed | [258] |
| 8 | C18 | 40 | 0.01 potassium dihydrogen orthophosphate : Acetonitrile (60:40) pH 3.0 | 1.0 | 260 | 2.38 | Impurities used | No | Higher proportion of Acetonitrile | | [88] |
| 9 | C18 | | 0.1 M orthophosphoric acid, pH 3.5–Acetonitrile (85:15) | 1.0 | 280 | 5.73 | No | No | | Animal tissue & eggs<br><br>With CIP (Rt 6.99) and other FQs | [259] |
| 10 | C18 | | Acetonitrile:0.005 M tetrabutylammonium bromide (10:90) pH adjusted to 2.0 with phosphoric acid | 2.0 | 254 | 2.4 | | | High flow rate.<br><br>Also pH 2.0 is not healthy for C18 column | | |
| 11 | C18 | | Acetonitrile: PA: Tetrabutylammonium hydroxide (100: 1.67:15) in 1 L water | 2.0 | FL | 2.3 | No | No | IS (A-56619) Rt 4.0<br><br>CIP (Rt 2.7) | FL detection in RM | [121] |
| 12 | C18 | | (3:1:10) Methanol: Acetonitrile: 0.4M citric acid | 1.0 | 275 | 8.67 | No | No | Higher ratio of organic phase | In body fluids | [156] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | C18 | | Acetonitrile: 0.5 M potassium dihydrogen orthophosphate buffer pH 4.5 with triethylamine (30:70) | 0.9 | 289 | 9.9 | No | No | Higher ratio of organic phase | With metronidazole | [260] |
| 14 | Anion exchange | | Acetonitrile-phosphate buffer0.05M, pH 7.0 (20:80) | 1.2 | 273 | 6.1 | No | No | Anion Exchange<br><br>Higher ratio of organic phase in RP | Serum, urine | [261,262] |
| 15 | C18 | | 18 mM potassium dihydrogen phosphate with 0.13mM heptane sulphonic acid, methanol and concentrated phosphoric acid (700:300:1) | | UV (278) & FL | 5.7 | | | Higher ratio of organic phase in RP<br><br>FL detection | Serum<br><br>With CIP, OFL & other FQs | [157] |
| 16 | C18 | | Methanol: phosphate buffer (30:70) | 2.0 | UV & FL | 5.1 | No | No | Higher proportion of organic phase in RM | Plasma, urine<br><br>With ion-pair reagent | [263] |
| 17 | C18 | | Phosphate buffer pH 4.6: Acetonitrile (45:55) | 1.0 | 254 | 4.51 | No | No | Higher proportion of organic phase in RM | With metronidazole | [264] |
| 18 | C18 | | Buffer pH 4: Acetonitrile (82:18) | 1.0 | 290 | 3.4 | Acid-base 0.1M HCl/NaOH for 90min, 60°C for 60min, 3% $H_2O_2$ | No | Higher proportion of organic phase in RM | With metronidazole | [265] |
| 19 | C18 | | Acetate buffer pH 4.8: Acetonitrile (85:15) | 2.0 | FL | 2.8 | No | No | FL detection | Plasma, tissue<br><br>With Pefloxacin and some metabolites | [266] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 | C18 | | Gradient<br><br>Acetonitrile: water: buffer with disodium hydrogen phosphate, tert-Butyl alcohol iodide adjusted to pH 9.7 with Triethylamine | 2.0 | 270 | 4.0 | | | pH 9 is not recommended for C18<br><br>Gradient elution | Plasma, urine<br><br>With Pefloxacin and some metabolites | [267] |
| 21 | C8 | ambient | Methanol: 0.01% Trifluoroacetic acid (25:75) | 1.2 | FL | 6.9 | No | No | FL detection used in RM | Plasma Enrofloxacin as IS<br><br>Details of mobile phase not clear | [268] |
| 22 | C18 | RT | Water: methanol: Acetonitrile (90:5:5) | 1.0 | FL | | | | FL detection | With CIP & OFL<br><br>Blood, serum, urine | [159] |
| 23 | C8 | RT | Acetonitrile: methanol : phosphate buffer (pH 2.5) (19:3:78) | 1.2 | FL | 6.0 | No | No | FL detection | Human tissues & plasma | [269] |
| 24 | C18 | | Acetonitrile-0.025 M orthophosphoric acid adjusted to pH 3.0 with Tetrabutylammonium hydroxide (11:89) | 1.5 | FL | 2.8 | No | No | FL detection | Serum, urine<br><br>with CIP | [160] |
| 25 | C8 | | Acetonitrile:<br><br>0.005M (pH 2.5) phosphate buffer (80: 20) | 2.0 | 282 | 4.5 | No | No | High proportion of organic phase in RM | Plasma, urine | [270] |
| 26 | C18 | 40 | Methanol : 0.1 M perchloric acid and 0.02 M triethylamine (30:70) | 1.0 | FL | 2.0 | RT<br><br>Freeze-thaw | | High proportion of organic phase in RM<br><br>FL detection<br><br>elevated temp | Plasma urine | [271] |
| 27 | C18 | 40 | Methanol: 0.005 M sodium laurylsulphate (2 : 1, v/v) adjusted to pH 2.5 with 85% phosphoric acid | 0.6 | 284 | 6.5 | No | No | Elevated temp | Plasma | [272] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Higher ratio of organic phase in RM | | |
| 28 | C18<br><br>Guard cartridge | 30 | Acetonitrile: 0.01 M phosphate buffer (pH 2.5) containing 0.001 M triethylamine (11:89) | 1.0 | 279 | 1.9 | | | 70% recovery | Serum<br><br>Used guard cartridge instead of analytical column | [273] |
| 29 | C18 | 40 | Acetonitrile: phosphate buffer, pH 3.0, 25mM (15:85) | 1.0 | FL | 4.33 | RT<br><br>Freeze-thaw | No | Elevated temp<br><br>FL detection | CIP as IS | [274] |
| 30 | C8 | | Acetonitrile: methanol: citric acid (0.4mol/L) (7:15:78) | 1.2 | 275 | 7.812 | Solution stability 6months of refrigeration | No | 2 components in organic phase of mobile phase in RM & higher proportion | Serum & pharmaceuticals<br><br>Enoxacin (Rt 6.571)<br><br>CIP(Rt 8.566)<br><br>OFL (Rt 7.266)<br><br>HCT as IS (Rt 4.272) | [126] |
| 31 | C18 | | 5.3mM phosphate buffer of pH 3.5: Acetonitrile (60:40) | 0.5 | 278 | 2.11 | No | No | Higher proportion of Acetonitrile in RM | tablets | [275] |
| 32 | C18 | 25 | Gradient( stepwise)<br><br>0.05 M Sodium dihydrogen phosphate (pH 2.5)–Acetonitrile | 1.3 | 275 | 15.1 | No | No<br><br>Some peaks are of known photodegradants | Gradient elution in RM<br><br>Long run/ retention time | With known impurities | [276] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 33 | C18e | ambient | Methanol: 0.025M Potassium dihydrogen phosphate adjusted to pH 3 with Ortho phosphoric acid (20:80) | 4.0 | 290 | 1.6 | No | No | High flow rate, higher methanol.<br><br>Monolithic silica column | | [204] |
| 34 | C18 | | Methanol: Sodium dihydrogen phosphate: Acetonitrile (30:30:40) adjusted to pH 3.5 with Ortho phosphoric acid | 1.0 | 280 | 2.46 | No | No | High proportion of organic phase in RM | Plasma<br><br>With CIP (Rt 2.81 ) | [245] |
| 35 | C18 | | Water: methanol adjusted to pH 3 with phosphoric acid (65:35) + Tetrabutylammonium hydroxide | 1.0 | FL | 4.169 | No | No | High proportion of methanol<br><br>FL detection | Chicken tissue | [277] |
| 36 | C18 | | 0.01mol/L: Acetonitrile: Tetrahydrofuran (89:10:1) | 1.0 | 280 | 10.8 | No | No | use of Tetrahydrofuran | With H-NMR<br><br>Enoxacin as IS | [278] |

**Drug 5: Ofloxacin**

A detailed literature search revealed 54 HPLC methods, among which 6 reported methods used gradient or stepwise elution, 10 methods used fluorometric detection, 7 used MS detection, 5 methods needed rare or special columns, 1 method used column switching technique whereas 8 of the methods worked at elevated temperatures. Ofloxacin being racemic, many chiral chromatographic separations have been reported. The rest of the reported methods were found to be using a higher proportion of organic phase as compared to developed method.

## Table 3.13. HPLC methods reported in literature in case of Ofloxacin

| Sr. No. | HPLC Method variables | | | | | Retention time for drug peak (min) | Acid/ Base/ Neutral hydrolysis/ Oxidation/ Photolytic/ Thermal (Or stability) | Degradants/ Peak (Name and structure) | Comparison with Developed Method (DM) with Reported Method (RM) | Remarks | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Column | Column temp. (°C) | Mobile Phase | Flow rate (mL/ min) | Detection (FL/ UV) (nm) | | | | | | |
| 1 | C18 | ambient (25) | Acetonitrile: water with 10 mM Tert-butyl acetoacetate, 10 mM sodium dodecyl sulphate & 25 mM citric acid (35: 65), pH 3.4 | 1.3 | multiple wavelengths | 11.1 | | No | Higher proportion of Acetonitrile in RM | With OFL, CIP, lomefloxacin, nalidixic acid | [279] |
| 2 | C18 | | Buffer pH 3.0: Acetonitrile (80:20) | 1.0 | 300 | | | | Higher proportion of Acetonitrile in RM | With ornidazole | [280] |
| 3 | C18 | | Acetonitrile: buffer (40:60)<br><br>Buffer- 2g Na acetate, 2g Na citrate, 1mL Triethylamine in 850mL water, pH 4.5) | 1.5 | FL | 1.06 | Stability in infusion bags containing saline & glucose | No | FL detection for RM | OFL & pefloxacin ( different method parameters for each) | [110] |
| 4 | C18 | RT | Acetonitrile: dihydrogen phosphate buffer pH 3.0 (35:65) | 1.0 | 296 | 2.58 | 0.1 N HCl & NaOH, 10% $H_2O_2$, 50°C thermal | 2.4 min for acid & base hydrolytic degradant of OFL | Higher proportion of Acetonitrile in RM | With Satranidazole | [281] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | C18 | RT (25) | Methanol: potassium dihydrogen phosphate buffer + Triethylamine adjusted to pH 3 with phosphoric acid (55:45) | 0.8 | 270 | 4.246 | 1N & 5N HCl & NaOH, 6% $H_2O_2$, 40°C thermal, sunlight | No | Higher proportion of meth in RM | With ketorolac | [282] |
| 6 | LC-MS/MS (Penta fluorophenyl column) | | Gradient<br><br>Acetonitrile: water with 0.1% formic acid | 0.4 | MS | 3.24 | RT<br><br>Freeze-thaw cycles | No | MS detection<br><br>Gradient elution in RM | Plasma<br><br>Gatifloxacin as IS<br><br>No fragmentation data | [283] |
| 7 | C18 | | Acetonitrile: 0.25M potassium dihydrogen phosphate (80:20) with 0.5% Triethylamine adjusted to pH 2.5 with Ortho phosphoric acid | 1.0 | 320 | 2.19 | No | No | High proportion of ACN | With nitazoxanide | [284] |
| 8 | C18 | RT | 0.1M citric acid: methanol (75:25) | 2.0 | FL | 438 sec<br><br>(7.3) | No | No | Higher proportion of organic phase | Serum<br><br>With metabolites desmethyl OFL & OFL-N-oxide | [285] |
| 9 | C18 | | 18 mM potassium dihydrogen phosphate with 0.13mM heptane sulphonic acid, methanol and concentrated phosphoric acid (700:300:1) | | UV (278) & FL | 5.7 | | | Higher ratio of organic phase in RP<br><br>FL detection | Serum<br><br>With CIP, OFL & other FQs | [157] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | C18 | | Acetonitrile: methanol: water (6.5:2.5:1) | 1.0 | 313 | 3.99 | No | No | High proportion of organic phase in RM | With metronidazole | [286] |
| 11 | C18 | 40 | Methanol: 0.005M sodium lauryl sulphate (2:1) adjusted to pH 2.5 with phosphoric acid | 0.6 | 275 & 300 | 5.9 | No | No | High proportion of organic phase in RM | Plasma<br><br>With fenbufen and its active metabolite felbinac.<br><br>Two IS | [287] |
| 12 | C18 | | Water with 0.055% Triethylamine: Acetonitrile (80:20) adjusted to pH 3 with Ortho phosphoric acid | 1.0 | 284 | 6.5 | No | No | Higher proportion of Acetonitrile in RM | With Ornidazole | [288] |
| 13 | C18 | 45 | Acetonitrile: aqueous solution of ammonium acetate and potassium perchlorate pH adjusted to 3.5 with Ortho phosphoric acid (18:82) | 1.0 | 294 | 9.997 | No | Decarb OFL<br><br>(4.343)<br><br>9-pip OFL (8.13)<br><br>desmeth OFL (9.06)<br><br>N-oxide (14.275) | Higher proportion of Acetonitrile and elevated temp in RM | With degradants desmethyl OFL, decarboxy OFL, OFL-N – oxide, 9-piperazino OFL | [98] |
| 14 | C18 | ambient | Acetonitrile: water with 0.04M PA, 0.1M Sodium dihydrogen phosphate, 0.5% sodium dodecyl sulphate and 0.005 M acetyl hydroxamic acid (42:58) | 1.0 | FL | | No | No | Multicomponent aqueous phase, higher proportion of Acetonitrile, FL detection in RM | | [165,166] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | C18 | 25 | Methanol: citric acid-Triethylamine buffer (30:70) | 1.0 | 289<br><br>MS | | | | Higher proportion of organic phase | Fenton Oxid | [289] |
| 16 | BEH - C18<br><br>UPLC | | Methanol: 0.1%formic acid in water (18:82) | 1.0 | 294 | | | | UPLC column | | [290] |
| 17 | C18 | 60 | Gradient<br><br>Ammonia Tetrabutylammonium hydroxide buffer: Acetonitrile | 1.0 | 294 | 20.0 | 5M NaOH & HCl, 30% $H_2O_2$,<br><br>105C | | Elevated column temp<br><br>Gradient elution<br><br>Long run ( 30-40min) | 5 known impurities taken (EP) | [99] |
| 18 | C18 | 45 | Acetonitrile: 50mM ammonium acetate buffer with 20mM trifluoroacetic acid, pH 3.3 (16:84) | 1.0 | 294 | 15.68 | 1N HCl & NaOH, 80°C<br><br>Dry heat 80°C<br><br>UV, 30% $H_2O_2$ for 5d | 11 peaks not named ( Rt from 2.25 to 14.16) | Elevated temp<br><br>Longer run<br><br>Higher proportion of Acetonitrile | | [291] |
| 19 | C18 | | Gradient<br><br>Methanol: 0.05% formic acid | 0.2 | MS | 13.44 | Photolytic | | MS detection<br><br>Gradient elution | many fragments | [292] |
| 20 | C18 | 30 | Gradient<br><br>Eluents-<br><br>Acetonitrile/Methanol/0.025M TBA·Cl/TFA | 250µl/min | FL | 4.5 | Autosampler for 15hr<br><br>Freeze-thaw | No | Plasma<br><br>With MOX Rt 13), CIP (Rt 6.6) and IS | FL detection and gradient elution in RM | [135] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | A: 75/25/899/1 B :150/50/799/1 both at pH 3.5 | | | | | | (sarafloxacin Rt 10.6) | | |
| 21 | C8 | 50 | Acetonitrile: citrate buffer pH 4.8 (15:85) | 1.5 | FL | 4.9 | Freeze-thaw | No | Plasma<br><br>N-allyl pefloxacin as IS (Rt 10.8) | Elevated temp<br><br>FL detection | [293] |
| 22 | C18<br><br>Chiral | RT | Chiral mobile phase with additive<br><br>phenylalanine, Copper(II) sulfate: methanol (86:14) | 1.0 | FL | S (13.0)<br><br>R (15.8) | No | No | Rat liver<br><br>CIP as IS (22.0) | Chiral separation<br><br>FL detection | [294] |
| 23 | C18 | | 0.45%Triethylamine: Acetonitrile adjusted to pH 2.3 with Ortho phosphoric acid (83:14) | 1.0 | 295<br><br>MS | 4.2 | UV | 9.6<br><br>20.8 | | | [295] |
| 24 | C18 | ambient | (0.04M) 1.9ml PA with 1.4g Tetrabutyl ammonium iodide, 300 ml methanol, 700ml water; pH 2.2 | 1.0 | 278 | 6.5 | | | Serum<br><br>NOR(Rt7.0), CIP (Rt7.5) & peloxacin (Rt 6.5)<br><br>IS DL8357 (Rt 4.1) | High proportion of organic phase | [123] |
| 25 | C18 | | Acetonitrile: water: Triethylamine (25:75:1), pH adjusted to 6 with PA | 1.0 | 300 | 3.5 | RT for 6 days | No | Simultaneous with:<br><br>CIP (Rt 2.7)<br><br>Tinidazole (Rt 4.5) | Higher proportion of organic phase as compared to DM | [128] |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Ornidazole (Rt 5.8) | | |
| 26 | C18 Waters Acc Q tag (amino acid analysis) | 30 | Gradient<br><br>Methanol: Acetonitrile: TBA.Cl : Trifluoroacetic acid | 1.0 | FL | 16.7 | No | No | Gradient elution,<br><br>Fluorimetric detection and special column in RM<br><br>Run/ Retention time too long | Aqueous & vitreous humor<br><br>With MOX<br><br>Sarafloxacin as IS | [227] |
| 27 | C18 | 45 | Ammonium acetate, potassium perchlorate buffer: Acetonitrile (82:18)<br><br>Nanocomposites | 1.0 | 294 | 10.01 | photocatalytic advanced technique | 9-piperazino OFL (7.99) & desmethylOFL (9.68) | Elevated temp | with CIP | [100] |
| 28 | C18 | | 2mM phosphate buffer: Acetonitrile (70:30), pH adjusted to 3.5 with Ortho phosphoric acid | 1.0 | 293 | 2.1 | No | No | With ornidazole (5.5) & IS GATI (2.5) | Higher proportion of Acetonitrile | [296] |
| 29 | C18 | | Acetonitrile: methanol: 0.025M phosphate buffer, pH 3.0 (30 :10: 60) | 1.0 | 318 | 4.04 | No | No | with 2 isomers of ornidazole (5.82 & 6.77) | More components and proportion of organic component | [297] |
| 30 | C18 | 25 | 0.03m potassium dihydrogen phosphate buffer pH 3: Acetonitrile (55: 45) | 0.8 | 230 | 2.91 | No | No | with Rifampicin (4.87) | Higher proportion of Acetonitrile | [298] |
| 31 | C18 | 25 | Acetonitrile: methanol: 0.5% formic acid (23: 10: 67) | 0.6 | MS | 2.81 | Freeze thaw | No | Plasma<br><br>With cefixime | Higher proportion of Acetonitrile | [299] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | (3.28) & IS MOX (4.13) | | |
| 32 | C8 | | 0.5% Triethylamine, pH 3: Acetonitrile (73:27) | 1.2 | 303 | 2.3 | No | No | with Tinidazole (4.1) & IS ambroxol (5.1) | Higher proportion of Acetonitrile | [300] |
| 33 | C18 | 35 | Acetonitrile: 0.1% Triethylamine adjusted to pH 3 with Ortho phosphoric acid (15:85) | 1.0 | 294<br><br>MS | 1.69 | Freeze thaw<br><br>RT for 2h<br><br>-20°C for 7 days | No | rat plasma | Temperature control for column | [301,302] |
| 34 | C18 | RT | Acetonitrile: mixed phosphate buffer pH 4 (50:50) | 1.0 | 236 | 2.4 | No | No | with dexamethasone (4.0) | Higher proportion of Acetonitrile | [303] |
| 35 | ACE C18 | 30 | Methanol: 0.1% formic acid (40:60) | 1.0 | MS | | No | No | MS method | Higher proportion of organic phase<br><br>MS detection | [304] |
| 36 | C8 | | Methanol: 0.025mM potassium dihydrogen phosphate buffer (70:30) | 1.0 | 290 | 6.24 | No | No | With cefixime (3.60) | Higher proportion of organic phase | [305] |
| 37 | C18 | | Acetonitrile: methanol: 0.4M citric acid (60:30:10) | 0.6 | 304 | 3.122 | No | No | with nitazoxanide (5.902) | Higher proportion of | [306] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | multicomponent organic phase | |
| 38 | Cellulose (C4) | | Methanol (43.3), Acetonitrile (46.6), hexane (10) with small amounts of acetic acid (0.4), diethyl amine (0.2) | 1.4 | 285 | | No | No | Enantiomeric separation | Higher proportion of organic phase | [307] |
| 39 | C18 | 25 | Water with 10mM L-phenylalanine & 5mM copper sulphate: methanol (85.5: 14.5) | 1.0 | FL | S-(7.5)<br><br>R-(8.7) | No | No | Rabbit plasma<br><br>Enantiomeric separation<br><br>Marbo as IS (5.6) | FL detection | [308] |
| 40 | C18 | | Phosphate buffer pH 2.8: Acetonitrile (82:18) | 1.0 | FL | 5.89 | No | No | Human serum<br><br>Suitable for CIP(6.5) & peflox (6.4) | FL detection | [309] |
| 41 | Column switching (phenyl & C18) | | 0.5% sodium acetate pH 2.5: Acetonitrile ( 87: 13) | 1.0 | UV | apprx 13 | No | No | Human serum<br><br>Enoxacin as IS | Column switching | [310] |
| 42 | C8 | 35 | Gradient<br><br>0.1% formic acid: methanol: Acetonitrile | 1.0 | 311 | 6.38 | Freeze thaw<br><br>RT for 24h | No | Human plasma | Gradient elution | [311] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | -20°C for 15days | | With flavoxate (8.96) | | |
| 43 | C18 | 30 | Triethylamine: Acetonitrile: 0.3% Ortho phosphoric acid (0.02:20:80) | 1.0 | 290 | 6.15 | No | No | With metronidazole (3.42) | Higher proportion of ACN | [312] |
| 44 | C18 | | Methanol: 25mM phosphate buffer adjusted to pH 5.5 with Ortho phosphoric acid (40:60) | 1.2 | 290 | 7.8 | No | No | With cefixime (2.5) | Higher proportion of organic phase | [313] |
| 45 | Chiral cel OD-H | 25 | Hexane: ethanol :acetic acid (60: 40: 0.1) | 0.5 | 294 | | No | No | Enantiomeric separation | Chiral column & mobile phase | [314] |
| 46 | C18 | | Methanol: 0.01M Tetrabutylammonium bromide : 0.5M phosphate buffer (35:65:4, pH 2.5) | 1.0 | 294 | | | | human plasma | Higher proportion of organic phase | [315] |
| 47 | C8 | 25 | Acetonitrile: buffer (35:65) | 1.5 | 315 | | No | No | | Higher proportion of Acetonitrile | [316] |
| 48 | C18 | ambient | Tetrahydrofuran: 25mM phosphate buffer, pH 5 with Ortho phosphoric acid (25:75) | 1.2 | 294 | 1.927 | 3days RT | No | With nimorazole (2.9) | Tetrahydrofuran used | [317] |
| 49 | C18 | | Acetonitrile: 0.025M phosphate buffer,pH2.5 (14.5:85.5) | 1.2 | 294 | 12.533 | No | No | CIP as IS<br><br>SPE used | longer Rt | [318] |
| 50 | C18 | ambient | 15mM buffer: methanol: Acetonitrile: Triethylamine (66:24:10:1) | 1.0 | 289 | 6.03 | 6 wks in fridge | No | with CIP (3.01) | Higher proportion of organic phase | [319] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 51 | C18 | | Acetonitrile: phosphate buffer pH 6 (30:70) | 1.2 | 300 | 3.72 | RT for 8hr | No | With satranidazole( 6.13) | Higher proportion of Acetonitrile | [320] |
| 52 | C18 | | Water: Acetonitrile: Triethylamine (83:14:0.45) adjusted to pH 2.3 with Ortho phosphoric acid | 1.0 | 295 | 5.1 | spiked/ frozen tissue extracts | No | Chicken tissues | No stability indicating assessment | [321] |
| 53 | C18<br><br>NPS | | 15mM potassium dihydrogen phosphate buffer pH 2.7: methanol (94:6) | 0.6 | 270 | 3.7 | 0.1N NaOH, HCl & water, 3% $H_2O_2$, 254nm UV | OFL acid hyd degradant peak at 2.19 | With meropenem (1.6) | Special column | [322] |
| 54 | C18 | | Acetonitrile: phosphate buffer (85:15) | 1.0 | 300 | 8.508 | No | No | With tinidazole (3.308) | Higher proportion of Acetonitrile | [323] |
| 55 | Anion exchange | | 0.05M phosphate buffer pH 7: Acetonitrile (20:80) | 2.0 | 297 | 10.4 | No | No | With NOR as IS (6.7) | Anion exchange column<br><br>Higher Acetonitrile, flow rate | [324] |
| 56 | C8 | RT | 0.35M glacial acetic acid, 4.8mM SDS : Acetonitrile (60:40) | 0.25 | FL | 10 | No | No | rat tissues & serum, mucosa | FL detection | [325] |
| 57 | C18 | | Acetonitrile: 0.2M phosphate buffer pH 2.4 (20:80) | 1.0 | 294 | 2.9 | No | No | microspheres | Higher proportion of Acetonitrile | [326] |
| 58 | C18 | | Methanol : 0.01M oxalic acid (35:65) | 1.0 | 298<br><br>MS | | Photocatalytic degradation with $TiO_2$& $H_2O_2$ | No | | Higher proportion of organic phase | [327] |
| 59 | C18 | | 0.025 M phosphate buffer pH 2.2 : Acetonitrile (85.5:14.5) | 2.0 | 294 | | | | plasma | low pH | [328] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | CIP as IS | | |
| 60 | C18 | 35 | sodium acetate pH3: Acetonitrile (with Triethylamine 10mM) (70:30) | 1.0 | 280<br><br>FL | 1.5-1.7 | No | No | with CIP, NOR | High Acetonitrile | [329] |
| 61 | C18 | 25 | Acetonitrile: pH 5.8 ammonium acetate buffer (25:75) | 1.0 | 293 | 4.278 | refluxed for 8h for acid,alkaline hyd, 80°C for 24h. direct sunlight 4h | extra peaks seen only for photolytic degradation | with ornidazole | higher CAN | [330] |
| 62 | C18 | RT | Methanol: potassium dihydrogen phosphate buffer pH 2.5 (42:58) | 1.0 | 295 | | | | Chinese | Higher proportion of methanol | [331] |
| 63 | C18 | | Methanol: 60mM phosphate buffer pH 3.5 (40:60) | 1.2 | 285 | 6.0 | No | No | | Higher proportion of methanol | [332] |
| 64 | C18 | 45 | Methanol: 0.05% trifluoroacetic acid (38:62) | 1.1 | 290 | 3.8 | No | No | with MOX (8.0) | Higher proportion of methanol | [253] |
| 65 | C8 | ambient | Gradient<br><br>0.2% Hexanesulfonic acid, pH 2.7 with Orthophosphoric acid: Acetonitrile | 0.8 | 243 | 4.18 | No | No | with 3 other drugs in creams | Gradient elution | [333] |
| 66 | Chiral | | 50mM potassium dihydrogen phosphate buffer pH2.6: methanol (60:40) | 1.8 | 240 -260 | R-13.5<br><br>S- 12.0 | No | No | | Chiral / enantiomeric separation | [334] |
| 67 | C18 | ambient | 0.05M phosphate buffer pH 2.4: Acetonitrile (80:20) with 1- heptane sulfonic acid | 0.6 | FL | | No | No | Chicken tissues | Higher proportion of Acetonitrile | [335] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 68 | C18 | ambient | Methanol: Acetonitrile: 0.4M citric acid (3:1:10) | 1.0 | FL | 7.32 | No | No | Aqueous humour<br><br>With pipemidic acid as IS (4.82) | Higher proportion of organic phase | [336] |
| 69 | C18 | | 50mM phosphate buffer pH 2.6 adjusted with disodium dihyd phosphate: Acetonitrile (82:18) | 1.5 | FL | 7.0 | No | No | Plasma & urine | Higher proportion of organic phase | [337] |
| 70 | Resolvosil BSA 7 ( for enantiomers) | ambient | Phosphate buffer pH 8: Propan-2-ol (97:3) | 1.0 | FL | 5.2 (-enantiomer) & 7.5 (+ enantiomer) | No | No | 2 methods enantiomeric separation | Special Column | [338] |
| 71 | C18 | 40 | 0.2M phosphoric acid adjusted to pH 1.85 with tetraethyl ammonium hydroxide: Acetonitrile (80:20) | 1.5 | FL | 2.6 (-deriv)& 3.8 (+deriv) | No | No | | Derivatisation | [338] |
| 72 | C18 | | Potassium dihydrogen phosphate buffer pH 2.6 with Ortho phosphoric acid : Acetonitrile (82:18) | 0.8 | FL | 9.39 | No | No | Human hair<br><br>Also for CIP (9.97) & NOR (9.09) | FL detection<br><br>Higher Acetonitrile | [339] |
| 73 | C18 | | 0.025M Ortho phosphoric acid pH adjusted to 3 with Tetrabutylammonium hydroxide: Acetonitrile (9:1) | 1.5 | FL | 4.0 | No | No | In bile | FL detection | [340] |
| 74 | C18 | | 40% Acetonitrile, 1% Ortho phosphoric acid, 0.2% sodium lauryl sulphate | 1.2 | FL | 6.7 | No | No | | FL detection | [341] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Higher proportion of Acetonitrile | |
| 75 | C8 | ambient | Tetrahydrofuran: 50mM potassium dihyd phosphate adjusted to pH 2 with Ortho phosphoric acid: 1 M ammonium acetate (7.5: 92.5:1) | 1.0 | FL | 9.6 | No | No | Serum, urine<br><br>With metabolites | FL detection | [342] |

### 3.5 Data on HPLC Methods for FQs

Data on HPLC methods reported for multiple FQs was collected through a thorough literature survey for the purpose of prediction of Retention time using Multiple Linear Regression. Data has been tabulated in Table 3.14.

**Table 3.14 HPLC Data of Fluoroquinolones**

| Sr. No | Method | | | | | | | Name Drug/Related Substance | Rt min | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| | Column | Mobile Phase | | | pH of buffer | Flow rate (mL/min) | Column dimensions | | | |
| | | Methanol | ACN | Aq Phase | | | | | | |
| 1 | C18 | | 10 | 90(TBAH- phosphate buffer) | 3.0 | 2.0 | 15cm x 3.9mm, 10µ | Norfloxacin | 2.3 | [121] |
| | | | | | | | | Ciprofloxacin | 2.7 | |
| 2 | C18 | | 35 | 65 (TBAA- sod.dodecyl sulphate, citric acid) | 3.4 | 1.3 | 15cm x 4.6 mm, 5µ | Nalidixic acid | 4.54 | [279] |
| | | | | | | | | ofloxacin | 7.85 | |
| | | | | | | | | lomefloxacin | 10.49 | |
| | | | | | | | | norfloxacin | 11.10 | |
| | | | | | | | | ciprofloxacin | 11.92 | |
| 3 | C18 | | 42 | 38 (sod.dodecyl sulphate, phosphate) | | | 25cm x 4.6 mm, 7µ | ciprofloxacin | No data available | [165] |
| | | | | | | | | ofloxacin | | |
| 4 | C18 | | 53 | 47 | | 1.5 | 25cm x 4.6 mm, 7µ | Temafloxacin | 6 to 9 | [166] |
| 5 | C18 | | | 0.025M TBA·Cl/TFA | | 250µL/min | | Ofloxacin | 4.5 | [135] |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Gradient<br><br>Eluent A:<br><br>25<br><br>Eluent B: 150 | <br><br>75<br><br>50 | 899:1<br><br>799:1 | 3.5 (both A&B) | | 15cm x 2.1 mm, 3.5µ | Ciprofloxacin | 6.6 | |
| | | | | | | | | Sarafloxacin | 10.6 | |
| | | | | | | | | Moxifloxacin | 13.0 | |
| 6 | C8 | | 14 | 86 (1%TEA) | 3.0 | 1.0 | 25cm x 4.6 mm, 5µ | Pazufloxacin | 11.3 | [130] |
| | | | | | | | | Cip | 14.5 | |
| | | | | | | | | Lev | 12.8 | |
| 7 | C18 | | 20 | 80<br><br>(phosphate buffer) | 2.5 | 1.0 | 25cm x 4.6 mm, 5µ | Gatifloxacin | 10.8 | [343] |
| | | | | | | | | Sparfloxacin | 12.8 | |
| | | | | | | | | Moxifloxacin | 17.0 | |
| | | | | | | | | Levofloxacin | 6.0 | |
| 8 | C18 | | 88 | 12<br><br>(10mM phosphate buffer with 2mM TBA.Br) | 2.5 | 1.2 | 15cm x 4.6 mm, 5µ | Levofloxacin | 5.19 | [181] |
| | | | | | | | | Gatifloxacin | 8.93 | |
| | | | | | | | | Moxifloxacin | 13.45 | |
| 9 | C18 | Gradient<br><br>C | B | A<br><br>0.1% formic acid adj with TEA | 3.0 | 1.0 | 5.5cm x 4 mm, 3µ | Levofloxacin | 3.6 | [185] |
| | | | | | | | | Pazufloxacin | 6.0 | |
| | | | | | | | | Gatifloxacin | 9.8 | |
| | | | | | | | | Moxifloxacin | 10.3 | |
| | | | | | | | | Trovafloxacin | 10.7 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Norfloxacin | 4.4 | |
| 10 | C18 | | 43 | 57<br><br>(10mM sodium dodecyl sulphate, 10mM TBAA. 25mM citric acid) | 3.5 | 1.0 | 25cm x 4.6 mm, 5µ | Cinoxacin | 3.8 | [136] |
| | | | | | | | | Levofloxacin | 4.7 | |
| | | | | | | | | Ciprofloxacin | 5.5 | |
| | | | | | | | | Gatifloxacin | 6.4 | |
| | | | | | | | | Moxifloxacin | 7.0 | |
| | | | | | | | | Trovafloxacin | 8.4 | |
| 11 | C18 | | 25 | 75<br><br>(0.1%TEA) | 6.0 | 1.0 | 25cm x 4.6 mm, 5µ | Ciprofloxacin | 2.73 | [128] |
| | | | | | | | | Ofloxacin | 3.59 | |
| 12 | C18 | | 50 | 50 (0.1% TEA adj with PA) | 3.3 | 2.0 | 10cm x 4.6 mm, 5µ | Pefloxacin | 1.59 | [110] |
| | | | 40 | 60 (sod.acetate-citrate, TEA) | 4.5 | 1.0 | | Ofloxacin | 1.45 | |
| | | | | | | 1.5 | | Ciprofloxacin | 1.06 | |
| 13 | C18 | | 20 | 80 (0.3% TEA) | 3.3 | 1.0 | 12.5cm x 4 mm, 5µ | Pefloxacin | 2.56 | [170] |
| | | | | | | | | Lomefloxacin | 2.81 | |
| | | | | | | | | Gatifloxacin | 4.14 | |
| | | | | | | | | Levofloxacin | 2.37 | |
| 14 | C18 | 30 | | 70 (TBA.I, PA) | 2.2 | 1.0 | 25cm x 4.6 mm, 5µ | Pefloxacin | 6.5 | [123] |
| | | | | | | | | Ciprofloxacin | 7.5 | |
| | | | | | | | | Ofloxacin | 6.5 | |
| | | | | | | | | Norfloxacin | 7.0 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 15 | C8 | 15 | 7 | 78<br><br>(0.4mol/L citric acid) | | 1.2 | 25cm x 4 mm, 5µ | Enoxacin | 6.571 | [126] |
| | | | | | | | | Ofloxacin | 7.266 | |
| | | | | | | | | Norfloxacin | 7.812 | |
| | | | | | | | | Ciprofloxacin | 8.566 | |
| 16 | C18 | 3-4%THF | | 96-97% buffer | 3.0 | | 15cm x 3.9 mm | Enoxacin | 4.7 | [255] |
| | | | | | | | | Norfloxacin | 5.2 | |
| | | | | | | | | Ciprofloxacin | 6.4 | |
| | | | | | | | | Ofloxacin | 8.9 | |
| 17 | C18 | | 27 | 1.5% TEA: 33.8 mM $NaH_2PO_4$ (36.5:36.5) | 2.5 | 1.0 | | Levofloxacin | 2.85 | [344] |
| | | | | | | | | Moxifloxacin | 4.75 | |
| | | 17 | 12 | 0.7% TEA: 50 mM $NaH_2PO_4$ (35.5:35.5) | 2.5 | 1.0 | | Levofloxacin | 5.41 | |
| | | | | | | | | Ciprofloxacin | 6.19 | |
| 18 | C18 | | 20 | 80 (Phosphate buffer) | 3.0 | 1.0 | 25cm x 4.6 mm, 5µ | Marbofloxacin | 4.25 | [345] |
| | | | | | | | | Enrofloxacin | 5.75 | |

# *CHAPTER 4*

## *DRUGS OF RESEARCH INTEREST*

# 4. Drugs of Research Interest

## 4.1 Rationale for Selection of Drugs

Drug molecules comprise of structures that exhibit their own reactivity and sensitivity while present in solution, necessitating an elementary study to understand the influence of acids, bases, oxidising agents, light and elevated temperatures on structural integrity of molecules prior to attempting analytical method development. This way any possible effects on the measurement parameters due to unanticipated incidences on the structure that otherwise could lead to erroneous results could be predicted.

For the study, compounds from FQ group were considered as they can provide a scope of large number of analogues bearing functional groups widely distributed over basic FQ nucleus resulting in they exhibiting diverse physical and chemical properties.

Also, it has been surveyed from literature that diverse conditions have been applied for the study of their stability giving rise to outcomes widely different from various sources.

The criteria considered for selection of drugs were based upon the physicochemical properties primarily although their spectrum of activity was also considered to enable selection of extant drugs.

The chemistry of molecules is required to be well-understood while considering the development of analytical methods. A study of physicochemical parameters was considered while selecting molecules of research interest. In selecting FQs diverse molecules with regard to core viz. bicyclic, tricyclic that were available for selection for a study on comparative stability, when frequently used operating conditions while conducting analytical studies was considered.

For the study, selected molecules from Fluoroquinolone group were considered as their number is vast, with diverse physical and chemical properties. Certain criteria were considered for their selection to meet objectives of study. The criteria considered for selection were:

### 4.1.1 Spectrum of Activity

Class I: Fluoroquinolones that are given orally, but the use is restricted to urinary tract infections, e.g., **Norfloxacin (NOR)**, Pefloxacin (PEF)[346].

Class II: Fluoroquinolones having wider uses and meant for systemic indications, e.g., **Ciprofloxacin (CIP)**, **Ofloxacin (OFL)**, Fleroxacin (FLE), Enoxacin (ENO).

Class III: Fluoroquinolones having increased effectiveness against Gram-positive and atypical pathogens. e.g., **Levofloxacin (LEV)**, Sparfloxacin (SPA), Grepafloxacin (GRE).

Class IV: Fluoroquinolones having increased effectiveness against Gram-positive and atypical pathogens as well as anaerobes, e.g., **Moxifloxacin (MOX)**, Gatifloxacin (GAT), Trovafloxacin (TRO), Clinafloxacin (CLI).

It was considered to select a compound to represent each class of drug representing spectrum of activity, viz. Norfloxacin, Ciprofloxacin, Ofloxacin, Levofloxacin and Moxifloxacin from Class I, II, III and IV respectively[346].

### 4.1.2 Structure and Physicochemical Properties of FQs:

Chemistry of molecules is generally exploited while considering the development of analytical methods. Study of physicochemical parameters was also considered while selecting the molecules of research interest.

Ciprofloxacin, Norfloxacin and Moxifloxacin have a bicyclic core, while Ofloxacin and its levo isomer Levofloxacin have a tricyclic core as shown in Fig.4.1.

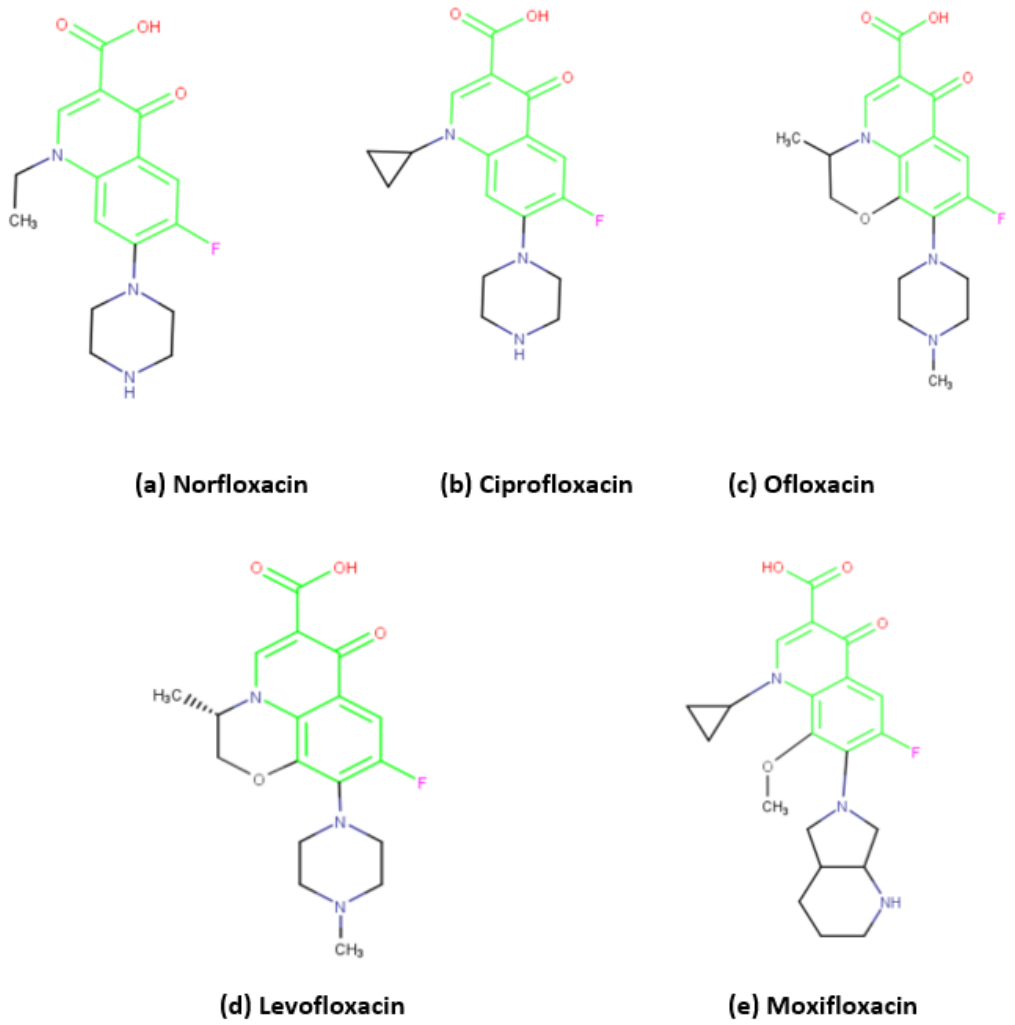



**Fig. 4.1. Structures of (a) Norfloxacin (NOR), (b) Ciprofloxacin (CIP), (c) Ofloxacin (OFL), (d) Levofloxacin (LEV) and (e) Moxifloxacin (MOX)**

As seen in Fig.4.1. the molecules of research interest are analogs of Fluoroquinolone carboxylic acids (6-fluoro-4-oxoquinoline-3-carboxylic acid). The two other substituents are alicyclic or heterocyclic bearing no additional chromogen nor active substituents that have a bearing on the quinolone ring[347]. All the five compounds have a similarity in

their pKa (5.4 to 5.6), as it being a factor that is considered critical for design of mobile phase of appropriate pH.

**Table 4.1. Physicochemical properties and Retention times of drugs in Method 1**

| FQ | pKa | clogP | Mobile Phase composition | Rt (min) | Remarks |
|---|---|---|---|---|---|
| CIP | 5.56, 8.77 | (-) 1.5264 | Methanol: phosphate buffer pH 3.0 (70:30), flow rate 1.25ml/min, 40°C column temp | 2.075 | Void volume is 2.0 ml and dead time is 1.6 min |
| LEV | 5.35, 6.72 | (-) 0.3426 | | 2.058 | |
| MOX | 5.49, 9.51 | (-) 0.9536 | | 2.133 | |
| NOR | 5.58, 8.77 | (-) 1.6498 | | 2.117 | |
| OFL | 5.35, 6.72 | (-) 0.3426 | | 2.067 | |

**4.1.2.1. LogP**:

*Log($c_{octanol}/c_{water}$) is logarithm of the partition coefficient of a substance between n-octanol and water*. clogP being an indicator of the molecular polarity and possibly a factor governing retention in partition chromatography was considered as a criterion for selection of appropriate Fluoroquinolones[348]. FQs with clogP spread over a narrow range were considered with constants of four among them varying from (-) 0.3426 up to (-) 1.6498. It was observed generally that the Rt increased with increase in clogP values confirming its role on the retention profile of the molecules.

**4.1.2.2. pKa**:

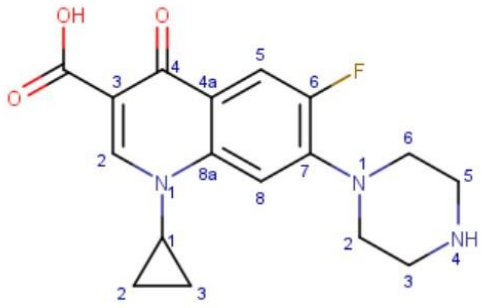
Another criterion considered for selection of drugs was pKa. *The acid dissociation constant (pKa) indicates the degree of ionization of molecules in solution at different pH values*[349].The FQs selected for study had varied values ranging closely from 5.35 to

5.58 (acidic) since the alkali sensitive silica columns remain stable over wide pH range in the acidic region.

Two sets of compounds (Levofloxacin vs Ofloxacin; Ciprofloxacin vs Norfloxacin) with identical pKa were selected for a comparative study on the effect of Ionizing ability (described by pKa) varying with Retention profiles.

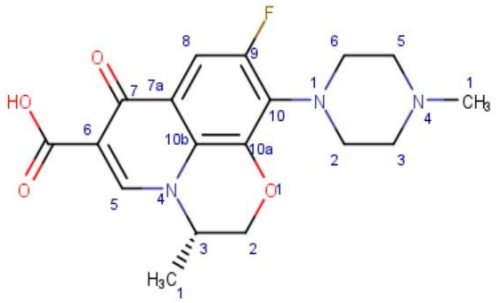
## 4.2 Drug Profiles

### 4.2.1 Ciprofloxacin

| | |
|---|---|
| General Name | Ciprofloxacin[30–32] |
| Chemical Structure |  |
| Chemical Name | 1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-yl quinoline-3-carboxylic acid |
| Molecular Formula | $C_{17}H_{18}FN_3O_3$ |
| Molecular Weight | 331.34 |
| Melting Point | 255-257°C |
| Description | Faintly yellowish to yellow crystal |
| Solubility | Soluble in dilute hydrochloric acid; practically insoluble in ethanol |
| pKa | 5.56, 8.77 |
| Drug Category | Anti-Infective Agents; Nucleic Acid Synthesis Inhibitors |
| Clinical Pharmacology | Ciprofloxacin is broad spectrum antibiotic, acts on bacterial topoisomerase II (DNA gyrase) and topoisomerase IV. It functions by inhibiting a type II topoisomerase (DNA gyrase) and topoisomerase IV, |

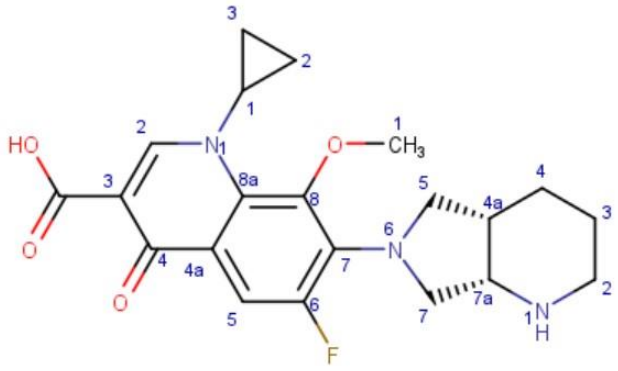
| | |
|---|---|
| | necessary to separate bacterial DNA, thereby inhibiting cell division[350,351]. |
| Pharmacokinetics | A maximum concentration of drug about 0.94 mg/L reaches in 0.81 hours after an oral dose (250 mg). Ciprofloxacin has a 70% absolute bioavailability. With a steady-state range following oral or intravenous dosage of 1.74 to 5.0 L/kg, the volume of distribution is substantial and reflects drug penetration into the majority of tissues[352].<br><br>About 27% of dose is excreted in the urine. |
| Toxicity | The oral LD50 in rats is >2000mg/kg. It has been documented that ciprofloxacin overdoses can cause acute renal damage. Ciprofloxacin's elimination half-life in older people ranges from 3.3 to 6.8 hours, as opposed to 3 to 4 hours in younger people[353]. There is very little data suggesting that ciprofloxacin is excreted in breast milk[354]. |

### 4.2.2 Levofloxacin

| | |
|---|---|
| General Name | Levofloxacin[30–32] |
| Chemical Structure |  |
| Chemical Name | (S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid |
| Molecular Formula | $C_{18}H_{20}FN_3O_4$ |
| Molecular Weight | 361.4 |
| Melting Point | 225-227 °C |
| Description | It is solid, light yellowish -white to yellow-white crystal or crystalline powder |
| Solubility | Freely soluble in glacial acetic acid, chloroform; sparingly soluble in water |
| pKa | 5.35, 6.72 |
| Drug Category | Anti-Bacterial Agents; Anti-Infective Agents |
| Clinical Pharmacology | Levofloxacin is a bactericidal fluoroquinolone antibiotic that inhibits bacterial DNA synthesis directly. Levofloxacin causes the breakdown of DNA strands in |

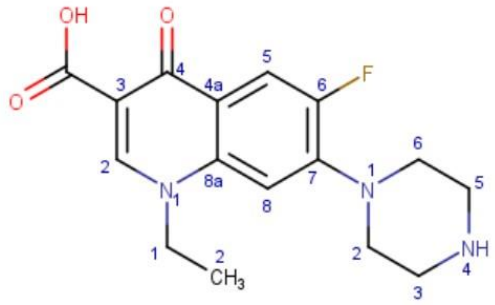
| | |
|---|---|
| | sensitive species by blocking DNA-gyrase, which prevents the relaxation of supercoiled DNA. |
| Pharmacokinetics | An oral administration of drug gives rapid absorption, with an oral bioavailability of approximately 99%. Levofloxacin is widely distributed in the body, with an average volume of distribution following oral administration between 1.09-1.26 L/kg (~89-112 L). Levofloxacin undergoes little hepatic metabolism in humans and is eliminated unchanged in the urine[355]. Approximately 87% of a single oral dose of levofloxacin was excreted unchanged in the urine after 48 hours and less than 4% was eliminated in the feces within 72 hours[356]. |
| Toxicity | The elimination half-life of levofloxacin in people with renal impairment ranges from 27 to 35 hours, depending on severity, compared to six to eight hours in healthy persons. This longer half-life implies that these patients require a dosage change. Levofloxacin has been linked to neurotoxicity, including status epilepticus that may not involve convulsions[357]. |

### 4.2.3 Moxifloxacin

| | |
|---|---|
| General Name | Moxifloxacin[30–32] |
| Chemical Structure |  |
| Chemical Name | 1-Cyclopropyl-6-fluoro-8-methoxy-7-(octahydro-6H-pyrrolo[3,4-b] pyridin-6-yl)-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid |
| Molecular Formula | $C_{21}H_{24}FN_3O_4$ |
| Molecular Weight | 401.4 |
| Melting Point | 238-242 °C |
| Description | Solid powder |
| Solubility | Soluble in water, ethanol, DMSO |
| pKa | 5.49, 9.51 |
| Drug Category | Antibacterial agent |
| Clinical Pharmacology | Moxifloxacin is bactericidal because it inhibits the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV. DNA gyrase is an important enzyme involved in bacterial DNA replication, transcription, and repair. Topoisomerase IV is an enzyme that plays a |

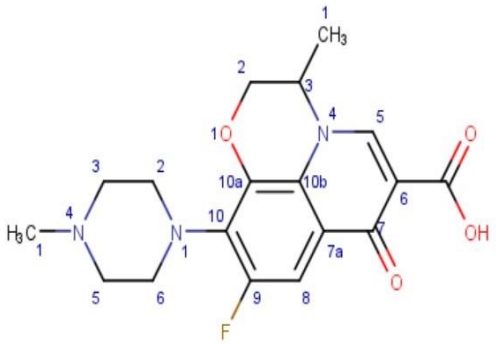
| | |
|---|---|
| | crucial role in the division of chromosomal DNA during bacterial cell division[350]. |
| Pharmacokinetics | Moxifloxacin is absorbed from the gastrointestinal tract. Moxifloxacin has oral bioavailability is approximately 90%.<br><br>Approximately 30-50% of moxifloxacin bound to serum proteins, independent of drug concentration. The volume of distribution of moxifloxacin ranges from 1.7 to 2.7 L/kg. Moxifloxacin is widely distributed throughout the body, with tissue concentrations often exceeding plasma concentrations.<br><br>Approximately 45% of an oral or intravenous dose of moxifloxacin is excreted as unchanged drug (~20% in urine and ~25% in feces). |
| Toxicity | CNS and gastrointestinal symptoms of an overdose include decreased activity, somnolence, tremor, convulsions, vomiting, and diarrhea. 100 mg/kg is the lowest intravenous fatal dosage for mice and rats[358]. |

### 4.2.4 Norfloxacin

| | |
|---|---|
| General Name | Norfloxacin[30–32] |
| Chemical Structure |  |
| Chemical Name | 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1H-quinoline- 3-carboxylic acid |
| Molecular Formula | $C_{16}H_{18}FN_3O_3$ |
| Molecular Weight | 319.33 |
| Melting Point | 227-228 °C |
| Description | It is solid, white to light-yellow crystalline powder |
| Solubility | It is freely soluble in glacial acetic acid, and very slightly soluble in ethanol, methanol and water. |
| pKa | 5.58, 8.77 |
| Drug Category | Anti-Bacterial Agents; Enzyme Inhibitors; Nucleic Acid Synthesis Inhibitors |
| Clinical Pharmacology | The bactericidal action of Norfloxacin results from inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, and recombination[350]. |

| | |
|---|---|
| Pharmacokinetics | Norfloxacin is rapidly absorbed after single dosages of 200 mg, 400 mg, and 800 mg. Approximately one hour after administration, the corresponding peak serum and plasma concentrations of 0.8, 1.5, and 2.4 g/mL are reached at the relevant dosages. In serum and plasma, the half-life of norfloxacin is 3–4 hours. Norfloxacin will reach steady-state concentrations within two days of treatment. The high rate of renal clearance (about 275 mL/min) demonstrates that renal excretion occurs via glomerular filtration and tubular secretion. Within 24 hours of medication administration, 26 to 32% of the provided dose is recovered in the urine as norfloxacin, and an additional 5 to 8% of the administered dose is recovered as six active metabolites with diminished antibacterial effectiveness. Norfloxacin's serum protein binding ranges between 10 and 15% [359]. |
| Toxicity | Norfloxacin has also been linked to rare but occasionally severe and even fatal cases of acute liver injury. |

### 4.2.5 Ofloxacin

| | |
|---|---|
| General Name | Ofloxacin[30–32] |
| Chemical Structure |  |
| Chemical Name | (RS)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl- 1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid |
| Molecular Formula | $C_{18}H_{20}FN_3O_4$ |
| Molecular Weight | 361.4 |
| Melting Point | 250-257 °C |
| Description | It is solid, off-white to pale yellow crystalline powder |
| Solubility | It is slightly soluble in water, alcohol, dichloromethane, and methyl alcohol but sparingly soluble in chloroform. |
| pKa | 5.35, 6.72 |
| Drug Category | Anti-Bacterial Agents; Anti-Infective Agents, Urinary; Nucleic Acid Synthesis Inhibitors |
| Clinical Pharmacology | Ofloxacin acts on DNA gyrase and topoisomerase IV, enzymes which, like human topoisomerase, prevents the excessive supercoiling of DNA during replication or transcription. By inhibiting their function, the drug thereby inhibits normal cell division[350]. |

| | |
|---|---|
| Pharmacokinetics | Ofloxacin has approximately 98% bioavailability. Less than 10% of a single dose of ofloxacin is metabolized. Ofloxacin is mainly eliminated by renal excretion, where between 65% and 80% of an administered oral dose of ofloxacin is excreted unchanged via urine within 48 hours of dosing. About 4-8% of an ofloxacin dose is excreted in the feces and the drug is minimally subject to biliary excretion[360]. |
| Toxicity | Ofloxacin has also been linked to rare but occasionally severe and even fatal cases of acute liver injury[361]. The time to onset is typically short (2 days to 2 weeks) and the presentation is often abrupt with nausea, fatigue, abdominal pain and jaundice. |

# *CHAPTER 5*

# *EXPERIMENTAL*

# 5. Experimental

## 5.1 Equipment and Chemicals

### 5.1.1 Instruments and Equipment:

HPLC (Jasco make) was used for development and validation of newer analytical methods, analyse market samples and for estimation of drugs after subjecting them to stress. For characterization of drug and impurities in stress-induced samples, Triple Quadrupole LC-MS system of Agilent Technologies (Courtesy BITS Goa Campus) was used. Details of instruments used in the research work have been listed in Table 5.1.

**Table 5.1: List of Instruments/ Equipment**

| Instruments | Source and Specifications |
|---|---|
| HPLC System | Jasco, LC- 4000 series quaternary pump system (PU-4180), an online degasser, an auto- sampler (AS-4050), a column temperature controller (CO- 4061) and a diode array detector (MD- 4010).<br>Software: Jasco Chrom NAV software |
| Column | PhenomenexRP-C-18 (4.6 ×250 mm, 5 µm) |
| Sonicator | Citizone Ultrasonic cleaner |
| pH meter | Digital pH Meter, Labtronics, LT- 10 |
| Electronic balance | Wensar Digital Electronic Balance MAB 220 |
| HPLC water purification system | Bio- age water purifier |
| Hot air oven | Universal |
| Constant temperature water bath | Tempo Single Phase TI 241B |
| LC-MS | 6400 Series Triple Quadrupole B.08.00 (B8023.5 SP1) of Agilent Technologies |

### 5.1.2 Chemicals and Reagents:

Chemicals and reagents used for the study:

- Methanol (HPLC grade)
- Acetonitrile (ACN, HPLC grade)
- Water (HPLC grade)
- Potassium dihydrogen ortho-phosphate ($KH_2PO_4$, AR grade)
- Sodium dibasic hydrogen phosphate ($NaH_2PO_4$, AR grade)
- Ortho phosphoric acid (OPA, HPLC grade)
- Triethylamine (TEA, HPLC grade)
- Formic acid (AR grade)
- Hydrochloric acid (HCl, AR grade)
- Sodium hydroxide (NaOH, AR grade)
- Hydrogen peroxide ($H_2O_2$, AR grade)

### 5.1.3 Drug Samples:

Ciprofloxacin (CIP), Levofloxacin (LEV), Moxifloxacin (MOX), Norfloxacin (NOR) and Ofloxacin (OFL) used for the study were obtained as gift samples from Abaris Healthcare Pvt. Ltd., Ahmedabad, Gujarat, India.

### 5.2 Exploratory Trials: Application of existing methods to other FQs.

Performance of Pharmacopeial methods on analysis of FQs, CIP, LEV, NOR, MOX and OFL were assessed.

#### 5.2.1 Procedure for Preparation of Solutions (CIP):

**Preparation of Standard Stock Solutions of CIP:**

A quantity of 25 mg of CIP was weighed accurately into a 25 mL volumetric flask and dissolved in methanol with sonication and volume made up with methanol to obtain a concentration of 1000 µg/mL (1 mg/mL).

**Preparation of Standard Stock Solutions of LEV, MOX, NOR and OFL:**

Stock solution of each drug was prepared in a similar way as in case of CIP above.

**Preparation of Working Standard Solutions of CIP:**

An aliquot of 0.2 mL of stock solution was diluted to 10 mL to obtain a final concentration of 20 µg/mL.

**Preparation of Working Standard Solutions of LEV, MOX, NOR and OFL:**

Working Standard solution of each drug was prepared in a similar way as in case of CIP above to obtain final concentrations of 20 µg/mL.

#### 5.2.2 Procedure for Preparation of Mobile Phase:

Appropriate volume of Orthophosphoric acid (OPA) required to prepare 0.1 % OPA was used to prepare 1000 mL.

Aqueous (0.1 % OPA) as well as organic components (methanol) of mobile phase were filtered through 0.45 µ membrane filters to remove any particulate matter and then sonicated for 15 min, for degassing.

### 5.2.3 Trials

For initial study, mobile phase comprising of Methanol: 0.1% OPA (30:70) was used for development, at a flow rate of 2 mL/min, with column temperature maintained at 50 °C, as non-buffered hydroalcoholic type mobile phase, and flow rate at highest mode with elevated column temperatures was used. The results of the study are presented with Rt of peaks from concerned chromatograms and system suitability parameters in Table 6.1 (pp 163)

Successively the trials involving use of Mobile Phase of Methanol: 0.1% OPA (40:60), flow rate of 2 mL/min, and column temperature of 50 °C was considered for the study.

The method using Mobile Phase of Methanol: 0.1% OPA (70:30), flow rate of 1 mL/min, and column temperature 40 °C showed higher tailing and low N and did not fall in the acceptance criteria for LEV and OFL. The results of the study are presented with Rt of peaks from concerned chromatograms and system suitability parameters in Table 6.2 (pp 164)

## 5.3 Method Development and Validation.

Experimental parameters such as flow rate, pH along with change in proportion of organic phase and buffer were explored and system suitability factors used to evaluate results.

### 5.3.1. Method 1

#### 5.3.1.1 Procedure for Preparation of Solutions for Method 1:

**Preparation of Stock and Working Standard Solutions of FQs:**

Standard stock solutions of 1000 µg/mL concentration were prepared for each of the FQs as per procedure provided in section 5.2.1 (pp 121). From the stock solutions of each FQ,

a working standard solution of the corresponding FQ was prepared resulting in final concentration of 20 µg/mL for each drug.

**5.3.1.2 Procedure for Preparation of Mobile Phase:**

Phosphate buffer (20 mM) was prepared by dissolving 2.72 g of Potassium dihydrogen orthophosphate in water, OPA was then used to set required pH and the volume made up to 1000 mL.

Aqueous as well as organic components of mobile phase were filtered through 0.45 µ membrane filters to remove any particulate matter and then sonicated for 15 min for the purpose of degassing.

**5.3.1.3 Development of Method 1**

**Trials:** Mobile phase proportion was varied from 70:30 (Methanol: phosphate buffer) to 40:60 (Methanol: phosphate buffer). The effect of pH of phosphate buffer was tried at 2.7, 3.0 and 3.3. Trials were conducted with flow rates of 1.0 and 1.25 mL/min with detection wavelength set at 294 nm.

Effect of mobile phase composition (Methanol: Phosphate buffer pH 2.7) and flow rates (1.0 mL/min and 1.25 mL/min) on retention time and system suitability are tabulated in Table 6.3 A and B (pp 165) and Table 6.4 (pp 166).

The outcome of change in pH of mobile phase composed of Methanol: Phosphate buffer (70:30) at 1.25 mL/min flow rate is reported in Table 6.5 (pp 167).

The effect of mobile phase adjusted to pH 3.0, at 1.25 mL/min flow rate, on retention and system suitability is presented as Table 6.6 (pp 168)

The effect of column temperature (30 °C, 40 °C, 50 °C and 60 °C), and use of loop volumes of 10 and 20 µL is tabulated as Table 6.7 (pp 169) and 6.8 (pp 170) respectively.

From the trials, it was observed that Methanol: Phosphate buffer pH 3.0 in the proportion 70:30 provided best results with regards to retention and system suitability. Flow rate of 1.25 mL/min and detection wavelength of 294 nm was selected. Column temperature was maintained at 40 °C.

Method 1 was applied to selected FQs of research interest using isocratic mobile phase system comprising of Methanol: Phosphate buffer pH 3.0, in the proportion 70:30 for development, at a flow rate of 1.25 mL/min, with detection wavelength of 294 nm and column temperature of 40 °C. The method parameters have been tabulated in Table 6.9 (pp 172) and representative chromatograms of each of the five selected FQs, namely, CIP, LEV, MOX, NOR and OFL, presented in Fig. 6.1, 6.2 and 6.3 (pp 172,173).

#### 5.3.1.4. System Suitability and Validation for Method 1

System Suitability and Validation for Method 1 parameters were then assessed separately for each of the selected Fluoroquinolones as per ICH guidelines[4,362].

**System Suitability Parameters**:

**5.3.1.4.1 Retention Time:** *The time elapsed between sample introduction (beginning of the chromatogram) and the maximum signal given by the sample at the detector is called as the Retention Time ($t_R$).* The retention time is longer when the solute has higher affinity to the stationary phase due to its chemical nature.

Retention time was assessed by six replicate injections of FQs at a concentration of 20 µg/mL prepared as stated in section 5.3.1.1 (pp 122) and the results for CIP are tabulated

in Table 6.10 A & B (pp 174), Table 6.17 A & B (pp 179) for LEV, Table 6.24 A & B (pp 184) for MOX, Table 6.31 A & B (pp 189) for NOR and Table 6.38 A & B (pp 193, 194) for OFL.

**5.3.1.4.2 Column Efficiency:** *Column Efficiency is a quantitative measure of the quality of separation*. The determination of column efficiency is an essential step while establishing system suitability of the analytical method for a drug by HPLC[363]. The efficiency of the column is evaluated by calculating number of theoretical plates per column:

$N= 16 (t/w)^2$

where,

t is the retention time;

w is the width at the base of the peak

Column efficiency was assessed by six replicate injections of each FQ at a concentration of 20 µg/mL prepared as stated in section 5.3.1.1 (pp 122) and the results for CIP are tabulated in Table 6.10 A & B (pp 173,174), Table 6.17 A & B (pp 179) for LEV, Table 6.24 A & B (pp 184) for MOX, Table 6.31 A & B (pp 189) for NOR and Table 6.38 A & B (pp 193, 194) for OFL.

The acceptance criterion for column efficiency was $N \geq 2000$.

**5.3.1.4.3 Peak Symmetry:** *The degree of peak symmetry is given by Symmetry Factor, also called "Tailing Factor"*. Good peak shape can be defined as a *symmetrical or gaussian peak* and poor peak shape can include both peak fronting and tailing. Gaussian peak shapes in chromatography indicate a well-behaved system and provide improved sensitivity (lower detection limits) and allow ease of quantitation.

Peak symmetry was assessed by six replicate injections of each FQ at a concentration of 20 µg/mL. The acceptance criterion for peak symmetry was a range from 0.8 to 1.2.

**Validation Parameters:**

**5.3.1.4.4 Linearity**

*Linearity is the linear range of detectability that obeys the Beer's Law*. It is dependent on the compound being analysed and the detector used. It was determined that the calibration curve was linear across the concentration range of 10 to 60 µg/mL of selected FQs.

**Preparation of Standard Solutions of CIP:**

Standard Stock solution of 1000 µg/mL concentration was prepared for CIP as per procedure provided in section 5.2.1 (pp 121). Aliquots were taken from stock solution of CIP, as per volumes shown in Table 5.2, and diluted to give working standard solutions of desired concentration. These resultant solutions were injected into the chromatograph under optimal conditions. The peak area versus concentration relationship was plotted to establish the regression equation, validated through acceptable correlation coefficient.

**Table 5.2 Preparation of Standard Solutions for Linearity Studies**

| Volume of CIP from Stock in mL | Final Volume made up to (mL) | Resultant Concentration (µg/mL) |
|---|---|---|
| 0.1 | 10 | 10 |
| 0.2 | 10 | 20 |
| 0.3 | 10 | 30 |
| 0.4 | 10 | 40 |
| 0.5 | 10 | 50 |
| 0.6 | 10 | 60 |

**Standard Solutions of LEV, MOX, NOR and OFL:**

Standard Solutions of LEV, MOX, NOR and OFL were prepared in a way similar to CIP standard solutions as per Table 5.2 (pp 126) and the resultant solutions were injected into the chromatographic column under optimal chromatography conditions.

The results of linearity studies for CIP have been reported in Table 6.11 (pp 175), Fig. 6.4 (pp 175). Linearity study data for LEV is presented in Table 6.18 (pp 180), Fig. 6.5 (pp 180), whereas those of MOX, NOR and OFL have been tabulated in Table 6.25 (pp 184), 6.32 (pp 189) and 6.39 (pp 194) and Fig. 6.6 (pp 185), 6.7 (pp 190) and 6.8 (pp 194) respectively.

**5.3.1.4.5 Limit of Detection (LOD) and Limit of Quantification (LOQ):**

*The limit of detection (LOD or the detection limit, DL) is the lowest concentration at which the method can reliably identify or detect the analyte inside the matrix*. It is also known as the lowest concentration that can be reliably distinguished from background noise.

*The limit of quantitation (LOQ or the quantification limit, QL) is the lowest concentration of the analyte that can be reliably and quantitatively measured by the method*. A suitable precision and trueness must exist and be shown in order for something to be considered reliable.

The method's limit of detection (LOD) and limit of quantification (LOQ) were obtained from the linearity plot. The signal-to-noise ratio for selected FQs (CIP, LEV, MOX, NOR, OFL) was used to calculate LOD and LOQ. The S/N ratio for LOD was 3 and for LOQ it was 10.

The LOD and LOQ were calculated for each FQ from their respective linearity relationship plots.

**5.3.1.4.6 Precision**

*The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of a homogeneous sample*. It is considered at three levels: repeatability, intermediate precision and reproducibility.

**Repeatability:** *Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.*

The repeatability of the method was evaluated by repeatedly injecting six times a 20 µg/mL solution of the selected FQ (CIP, LEV, MOX, NOR, OFL). The percent RSD should not be more than 2 percent.

**Intermediate Precision:** *Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.*

The intraday and inter-day accuracy of intermediate precision were used to assess the performance of the method. Six repetitive injections of selected FQs (CIP, LEV, MOX, NOR, OFL), 20 µg/mL concentration, were used to investigate the intraday precision. The precision within and between days was investigated by assessing the equivalent concentration six times on the same day and six times on other days. The findings were expressed as a percent relative standard deviation. The percent RSD should not be more than 2 percent.

**Reproducibility:** *Reproducibility expresses the precision between laboratories* (in case of collaborative studies, usually applied to standardization of methodology).

The results of precision studies performed on 30 µg/mL of CIP have been reported in Table 6.12 (pp 176). Precision studies carried out on 25 µg/mL concentrations of LEV, MOX, NOR and OFL have been presented in Table 6.19 (pp181), 6.26 (pp 186), 6.33 (pp 191) and 6.40 (pp 195) respectively.

### 5.3.1.4.7 Accuracy

*Accuracy is the measure of how close the experimental value is to the true value*. Spiking technique was used to assess the accuracy of the procedure under consideration. To previously examined sample solutions of the FQ were added standard solutions of various concentrations at levels ranging between 80 - 120 percent, as shown in Table 5.3.

**Table 5.3 Preparation of Solutions for Accuracy Studies**

| Level | Level of addition of Standard (%) | Assay value (previously analysed samples) (µg/mL) | Amount of std spiked (µg/mL) | Volume of std added from Stock (mL) | Final conc. (µg/mL) |
|---|---|---|---|---|---|
| 1 | 80 | 25 | 20 | 0.20 | 45 |
| 2 | 100 | 25 | 25 | 0.25 | 50 |
| 3 | 120 | 25 | 30 | 0.30 | 55 |

The resultant solutions were subjected to chromatographic analysis using the optimised mobile phase. The percentage of concentration recovered and the percentage of RSD at each dose level of the FQ were calculated and compared. In each level, the responses were examined with three replicate injections and the results for CIP have been reported in Table 6.13 (pp 177), while that for LEV, MOX, NOR and OFL have been presented in Table 6.20 (pp 182), 6.27 (pp 187), 6.34 (pp 191) and 6.41 (pp 196) respectively.

**5.3.1.4.8. Robustness**

*Robustness/ruggedness is defined as a measure of an analytical procedure's ability to remain unaffected by small but deliberate variations in parameters listed in the procedure documentation and to provide an indication of its suitability during normal use.*

Testing the robustness was done by observing the impact of minor deliberate changes in operational variables like the composition of the mobile phase solution, pH of aqueous component, flow rate on the performance of the method etc. Chromatographic parameters, including pH of aqueous component in mobile phase (±0.2 units), proportion of mobile phase components (±2%), and flow rate (±0.2 mL/min) of mobile phase, were deliberately altered in the current study to investigate their effect on the method and its performance. Robustness was evaluated by calculating the percent RSD.

The effect of ±2% variation in the mobile phase proportion, namely the ratio of organic phase to aqueous phase, 72:28 and 68:32 of methanol: buffer as compared to 70:30 used in the proposed method, has been studied and the results reported in Table 6.14 (pp 177), 6.21 (pp 182), 6.28 (pp 187), 6.35 (pp 192) and 6.42 (pp 196) for CIP, LEV, MOX, NOR and OFL respectively.

The impact of change in pH of buffer to 2.8 and 3.2 as compared to pH 3.0 used in the optimized method (resulting in ±0.2 units variation) was investigated and has been tabulated in Table 6.15 (pp 178), 6.22 (pp 183), 6.29 (pp 187), 6.36 (pp 192) and 6.43 (pp 197) for CIP, LEV, MOX, NOR and OFL respectively.

Variation in mobile phase flow rate by working at 1.05 mL/min and 1.45 mL/min as compared to 1.25 mL/min used in the proposed method (±0.2 mL variation) was checked and the outcome has been presented in Tables 6.16 (pp 178), 6.23 (pp 183), 6.30 (pp 188), 6.37 (pp 193) and 6.44 (pp 197) for CIP, LEV, MOX, NOR and OFL respectively.

**5.3.1.4.9 Assay of Marketed Formulation**

The marketed formulations used for the study were as follows:

- CIP: Ciplox- 500 (batch no. SB60176) manufactured by Cipla Ltd.
- LEV: Leon 500 (batch no. LB60710) manufactured by Dr. Reddy's Laboratories Ltd.
- MOX: Mahoflox 400 (batch no. C5ABP010) manufactured by Mankind Pharma Ltd.
- NOR: Norflox- 400 (batch no. ACT6163) manufactured by Cipla Ltd.
- OFL: Zenflox- 400 (batch no. E1AH0004) manufactured by Mankind Pharma Ltd.

The determination of content of FQs (CIP, LEV, MOX, NOR, OFL) in the tablets was carried out by weighing 20 tablets. The mean weight was determined and the tablets crushed to fine powder. Accurately 25 mg of tablet powder was then weighed and transferred to a 25 mL volumetric flask containing methanol, sonicated for 30 min, and diluted up to 25 mL using methanol and filtered. The resulting stock solution of about

1000 µg/mL was further diluted to get sample solution of 25 µg/mL. A 20 µL volume of sample solution was injected into chromatograph, six times, under the conditions described earlier. The peak areas were measured at 294 nm and concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation.

The system suitability and results of validation study for Method 1 of the 5 selected FQs are summarized in Table 6.45 (pp 198).

The method was applied to stress induced samples as described in section 5.3.1.5.

### 5.3.1.5. Forced Degradation

LEV and CIP were subjected to stress degradation under conditions that produce degradation.

**Preparation of Solutions:**

Stock solutions of 1mg/mL concentration were prepared for each drug as mentioned in 5.3.1.1 (pp 122). Further these standard solutions were used to prepare test samples for exposure to such conditions normally a drug experiences during analysis.

**Hydrolytic Degradation under Acidic Conditions:**

An aliquot of 0.2 mL stock was withdrawn from the stock solutions, a volume of 2 mL of methanol was added and resultant solutions subjected to hydrolysis using 2 mL of hydrochloric acid of increasing molarity, namely, 0.1 M, 1 M and 5 M of HCl. The degradative studies were carried out at room temperature as well as elevated temperatures of 65 °C, after making up the volumes to 10 mL with water. At the end of 6 h, the solutions

were neutralised and volume made up to 25 mL. The resultant solution was used to investigate the stress withstanding ability of drug under hydrolytic conditions.

It was observed that lower concentrations of acid produced insignificant degradation evident through decreased peak areas, but the samples degraded further aided by conditions that produced higher stress seen as degradant peaks. These samples degraded under stronger stress conditions (with 5 M HCl, water bath at 65 °C for 6 h) and hence need to avoid use of such extreme conditions during method development.

**Hydrolytic Degradation under Basic Conditions:**

An aliquot of 0.2 mL stock was withdrawn from the stock solutions, volume of 2 mL of methanol was added and resultant solutions subjected to hydrolysis using 2 mL of sodium hydroxide solution of increasing molarity, namely, 0.1 M, 1 M and 5 M of NaOH. The degradative studies were carried out at room temperature as well as elevated temperatures of 65 °C, after making up the volumes to 10 mL with water. At the end of 6 h, the solutions were neutralised and volume made up to 25 mL. The resultant solution was used to investigate the stress withstanding ability of drug under hydrolytic conditions.

No degradation was observed under these conditions.

**Oxidative Degradation:**

Oxidative degradation was induced with 30% hydrogen peroxide as oxidising agent.

An aliquot of 0.2 mL was withdrawn from the stock solutions, volume of 2 mL of methanol was added and resultant solutions were subjected to oxidation using 2 mL of 30 % hydrogen peroxide solution. The degradative studies were carried out at room

temperature, after making up the volumes to 10 mL with water. At the end of 6 h, the solutions were used to investigate the stability indicating capability of proposed method.

Substantial degradation was observed with 30% $H_2O_2$ in 6 h.

**Thermal Degradation:**

The FQs were subjected to conditions conducive to thermal degradation by placing the drugs in powder form and as solutions in hot air oven, maintained at elevated temperatures of 65 °C and 100 °C, for 6 h.

**Dry Powder (Solid State)**:

The drug in powder form spread evenly on watch glass was placed in hot air oven at 65 °C and 100 °C, for 6 h. It was then used to make solution as stated in section 5.3.1.1 (pp 122). The resultant solutions were checked for degradation using optimised chromatographic conditions thereby investigating the ability of drug to withstand exposure at elevated temperatures (65 °C and 100 °C) for prolonged duration (up to 6 h).

**Drug Solution:**

Stock solutions were prepared as mentioned in section 5.3.1.1 (pp 122) and dilutions made in mobile phase to achieve a final concentration of 20 µg/mL. The solution was placed in a hot air oven at 65 °C and 100 °C, for 6 h. The solution was then assessed for degradation by injecting into the chromatograph under optimised conditions.

No degradation was observed for the drug exposed to elevated temperature either in dry powder form or as solution under applied conditions of elevated temperature.

**Photolytic Degradation:**

Preparation of stock solutions was done as mentioned in section 5.3.1.1 (pp 122). Dilutions were then made with mobile phase to achieve a final concentration of 20 µg/mL. The solution was placed in a hot air oven at 65 °C and 100 °C, for 6 h, 24 h and 7 days. The solution was then evaluated for degradation by injecting into the chromatograph under optimised conditions.

No degradation was observed for sample solutions under the conditions used for study.

Method 1 was applied satisfactorily to all selected FQs. For LEV additional peak was found where resolution with possible degradant peak did not appear satisfactory and was found merged with drug peak, as seen in Fig.6.9 (pp 199)

This drawback necessitated the development of new method that could resolve degradant peak satisfactorily from drug peak.

**5.3.1.6 Solution Stability of Drugs**

The stability of the FQs under the test conditions while in solution was assessed. From literature and forced degradation studies performed in the laboratory, it was evident that the FQs under investigation need to remain stable at least over the periods of handling the solution till their injections into the chromatograph in presence of light and atmospheric oxygen, in solutions made with the mobile phase used as in test method, adjusted to an acidic pH of 3.0.

**Table 5.4. Forced Degradation Studies**

| Stress | Drug | Stressing Agent | Strength of Acid/ Base/ Peroxide | Temperature | Duration | Remarks |
|---|---|---|---|---|---|---|
| Acid Hydrolysis | CIP, LEV | HCL | 0.1 N, 1 N, 5 N | 65 °C | 6 h | Degradation observed in the form of additional peak |
| Base Hydrolysis | CIP, LEV | NaOH | 0.1 N, 1 N, 5 N | 65 °C | 6 h | Additional peak not observed |
| Oxidation | CIP, LEV | $H_2O_2$ | 30 % | 65 °C | 6 h | Degradation observed in the form of additional peak |
| Thermal | CIP, LEV | Dry Heat | ____ | 65 °C, 100 °C | 6 h | Additional peak not observed |
| Photolytic | CIP, LEV | Daylight | ____ | RT | 7 days | Additional peak not observed |

**Preparation of Solutions:**

Stock solutions of 1mg/mL concentration were prepared for each drug as mentioned in 5.3.1.1 (pp 122). From this stock solution, aliquot of 0.2 mL was taken and volume made up to 10 mL with mobile phase used for Method 3, composed of ACN: 0.1% TEA adjusted to pH 3.0 with formic acid (85: 15).

**Stability Studies:**

Stability of 20 µg/ml solutions of drugs in mobile phase was studied over a period of 24 h, and up to 7 days, at room temperature. A comparison of degradation produced in solutions stored on laboratory platform, (exposed to daylight, but not in direct sunlight) and those protected from light was done.

At the end of duration of exposure, the samples were analysed using proposed optimized methods. Readings were taken in triplicate and average peak area used to calculate percentage of drug that remained undegraded.

The observations are reported in Tables 6.85 and 6.86 for CIP, 6.87 and 6.88 for LEV, 6.89 and 6.90 for MOX, 6.91 and 6.92 for NOR, 6.93 and 6.94 for OFL (pp 249 to 251).

### 5.3.2 Method 2: Method Development for Analysis of LEV in presence of its degradant

#### 5.3.2.1 Procedure for Preparation of Solutions for Method 2:

**Preparation of Stock and Working (Standard) Solutions of FQs:**

Stock solutions of 1000 µg/mL concentration were prepared for each of the FQs as per procedure provided in section 5.2.1 (pp 121). From the stock solutions of each FQ, a

working standard solution of the corresponding FQ was prepared resulting in final concentration of 20 µg/mL for each drug.

**Preparation of Stress Induced Samples:**

CIP and LEV, stress induced samples were prepared as per procedures stated in section 5.3.1.5 (pp 132). In the chromatograms, LDA represents the degradant produced through acid hydrolysis of LEV and LDO the degradant of LEV subjected to oxidizing conditions.

**5.3.2.2 Trials towards Development of Method 2**

A method with improved resolution for LEV and its degradants was proposed to be developed in order to meet the acceptance criteria with resolution (Rs) of 2.0 between the drug and degradant. Various trials were conducted with methanol as well as acetonitrile, combined in different ratios with both sodium and potassium phosphate buffers and orthophosphoric acid (OPA). Effect of triethylamine (TEA) was also studied along with effect of varying flow rates.

Table 5.5 presents results of the trials performed while developing Method 2.

**Table 5.5. Trials planned for Development of Method for LEV and its Degradants**

| Trial No. | Mobile Phases |
|---|---|
| 1 | Methanol and 0.1%OPA |
| 2 | Methanol and Phosphate buffer |
| 3 | Methanol and Phosphate buffer with 0.4% TEA |
| 4 | Methanol and Sodium phosphate buffer (20mM $Na_2HPO_4$) |
| 5 | Trials with 0.1% TEA |
| 6 | Potassium dihydrogen phosphate buffer (to compare Sodium and Potassium phosphate buffers) |

The outcomes of these trials have been reported in Table 6.46 (pp 200). Suitability of buffers was evaluated by comparing between Sodium phosphate and Potassium phosphate buffers. The results are presented in Table 6.47 (pp 203).

The effect of flow rate was assessed by comparing performance between flow rates of 0.8, 1.0 and 1.25 mL/min. The results are presented in Table 6.48 (pp 204).

The effect on performance of the method by varying the proportion of mobile phase components deliberately was studied and results presented in Tables 6.49 to 6.52 (pp 204, 205).

Based on the observations, Sodium phosphate buffer (20 mM) with 0.1% TEA adjusted to pH 3.0 with OPA was chosen as buffer, for mobile phase ratio optimized of methanol and buffer was 43:57. The flow rate used was 0.8 mL/min.

The result of study is presented in Table 6.53 (pp 206) and resultant chromatograms presented as Fig. 6.13 & 6.14 (pp 206, 207).

Method 2 was developed for LEV that was showing ability to resolve peaks of LEV from degradants, using methanol and phosphate buffer (pH 3.0) in the proportion 43:57 at a flow rate of 0.8 mL/min and with column temperature of 40 °C. The method was shown to separate acid and oxidative degradant of LEV satisfactorily.

**5.3.2.3 System Suitability and Validation of Method 2**

**5.3.2.3.1 Retention Time:**

Retention time was assessed with six replicate injections of FQs at a concentration of 20 µg/mL prepared as stated in section 5.3.2.1 (pp 137).

**5.3.2.3.2 Column Efficiency:**

Column efficiency represented by the number of theoretical plates (N) was assessed with six replicate injections of solutions (standard and stress induced sample) prepared as stated in section 5.3.2.1 (pp 137).

**5.3.2.3.3 Peak Symmetry:**

Peak symmetry was also assessed by the same process described in section 5.3.2.1 (pp 137).

**5.3.2.3.4 Resolution:**

*Resolution ($R_s$) is another measure of the quality of separation. The resolution between two adjacent peaks may be calculated from the retention times of the peaks of interest ($t_{r2}$ and $t_{r1}$) and the baseline width of the peaks ($w_1$ and $w_2$).* It is given by:

$$\mathbf{R_s = (t_{r2} - t_{r1}) / (0.5 \times (w_1 + w_2))}$$

Resolution between the drug and degradant peaks was used for evaluating the stability indicating capacity of proposed methods.

The results of System Suitability observed for six replicate injections have been presented in Table 6.54 (pp 207).

**Validation Parameters**

**5.3.2.3.5 Precision:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections each of LEV working standard solution of concentration 20 µg/mL and stress induced sample of LEV, on two different days. Results are presented in Table

6.55 (pp 208), 6.56 (pp 210) and 6.57 (pp 212).

**5.3.2.3.6 Linearity:**

Stock solution of 1000 µg/mL concentration was prepared for LEV as per procedure provided in section 5.2.1(pp 121). Aliquots were taken from stock solution of LEV, as per volumes shown in Table 5.6, and diluted to give standard solutions of 30-210 µg/mL concentration. These resultant solutions were injected into the chromatographic column under optimal chromatography conditions. The peak area versus concentration relationship was plotted to determine the regression equation and correlation coefficient.

**Table 5.6 Preparation of Standard Solutions of LEV for Linearity Studies**

| Volume of LEV from Stock in mL | Final Volume made up to (mL) | Resultant Concentration (µg/mL) |
|---|---|---|
| 0.3 | 10 | 30 |
| 0.6 | 10 | 60 |
| 0.9 | 10 | 90 |
| 1.2 | 10 | 120 |
| 1.5 | 10 | 150 |
| 1.8 | 10 | 180 |
| 2.1 | 10 | 210 |

The results of linearity studies for LEV have been presented in Table 6.58 (pp 213), Fig. 6.15 (pp 213).

**5.3.2.3.7 Limit of Detection (LOD) and Limit of Quantification (LOQ):**

The method's limit of detection (LOD) and limit of quantification (LOQ) were obtained from the linearity plot.

#### 5.3.2.3.8 Accuracy

Spiking technique was used to assess the accuracy of the procedure under consideration. To previously examined sample solutions of LEV were added standard solutions of various concentrations at levels ranging between 80 - 120 percent, as shown in Table 5.3 (pp 129).

The resultant solutions were subjected to chromatographic analysis using the optimised mobile phase. The percentage of concentration recovered and the percentage of RSD at each dose level of LEV were calculated and compared. In each level, the responses were examined with three replicate injections and the results for LEV have been reported in Table 6.59 (pp 214).

#### 5.3.2.3.9 Robustness

The robustness of the approach was assessed by observing the impact of minor changes in experimental variables like the composition of the mobile phase solution, pH of aqueous component, flow rate on the performance of the method. Chromatographic parameters - pH of aqueous component in mobile phase (±0.2 units), proportion of mobile phase components (±2%), and flow rate (±0.2 mL/min) of mobile phase, were deliberately altered in the current investigation to investigate their effect on the method and its performance. Robustness was evaluated by calculating the % RSD.

The effect of ±2% variation in the mobile phase proportion, namely the ratio of organic phase to aqueous phase, 41:59 and 45:55 of methanol: buffer as compared to 43:57 used in the proposed method, has been studied and the results reported in Table 6.60 (pp 215), for LEV and degraded samples containing degradants LDA and LDO.

The impact of change in pH of buffer to 2.8 and 3.2 as compared to pH 3.0 used in the optimized method (resulting in ±0.2 units variation) was investigated and has been tabulated in Table 6.61 (pp 215) for LEV and its stress induced samples. % RSD was calculated.

Variation in mobile phase flow rate by working at 0.6 mL/min and 1.0 mL/min as compared to 0.8 mL/min used in the proposed method (±0.2 mL variation) was checked and the outcome has been presented in Tables 6.62 (pp 216) for LEV and its degraded samples. Flow rate is expected to proportionately alter the retention time of the eluates.

**5.3.2.3.10 Assay of Marketed Formulation**

The proposed HPLC method was extended for the determination of LEV in tablets (Leon 500, batch no. LB80304, manufactured by Dr. Reddy's Laboratories Ltd.). Analysis was carried out using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 2.

The system suitability and validation results of Method 2 are summarised in Table 6.63 (pp 217).

Though the proposed Method 2 was found to be stability indicating, phosphate buffer a non-volatile component is not considered suitable for LC-MS systems. Hence a separate method development was proposed that would be LC-MS compatible and aid in studying stability profiles.

### 5.3.3. Method 3: Method Development for Analysis of CIP and LEV on LC-MS.

#### 5.3.3.1 Procedure for Preparation of Solutions for Method 3:

**Preparation of Stock and Working (Standard) Solutions of FQs:**

Stock solutions of 1000 µg/mL concentration were prepared for CIP and LEV as per procedure provided in section 5.2.1 (pp 121). From the stock solutions of each FQ, a working solution of the corresponding FQ was prepared resulting in final concentration of 20 µg/mL for each.

**Preparation of Stressed Samples:**

CIP and LEV, were subjected to stress as per procedures stated in section 5.3.1.5 (pp 132) and were used for developing a new stability indicating method.

LDA refers to the degradant produced upon acid hydrolysis of LEV and LDO to the degradant formed in oxidised sample of LEV.

#### 5.3.3.2 Trials towards Development of Method 3

Attempts were made for development of MS compatible methods for LEV that could be used for characterization i.e., identify degradants through Mass Spectrometry (LC-MS). Method 2 used phosphate buffer, a non-volatile buffer and hence not useful for LC-MS analysis. Non-volatile buffers and additives are not advised since they cause precipitation in the MS. Volatile buffer (like ammonium acetate) and use of appropriate additives (like formic acid, triethyl amine (TEA)) is required for LC-MS enabled methods.

**Trials:** Trials involved using acetonitrile (ACN) in combination with acetate buffer, formic acid, triethylamine, adjusted to varied pH conditions and in various proportions, and flow rates. Acetate buffer of differing strengths, from 20 to 50 mM were used.

Table 5.7 presents list of the trials conducted while developing Method 3.

**Table 5.7. Trials conducted to find suitable LC-MS compatible HPLC method for LEV and its degradants**

| Trial No. | Experimental Conditions (Mobile phase, pH, flow rate) | Chromatogram |
|---|---|---|
| 1 | ACN: acetate buffer, 20mM, pH 3.0, (20:80 and 30:70), 1.0ml/min | Fig. 6.16, 6.17& 6.18 |
| 2 | ACN: acetate buffer, 20mM, pH 6.0 (30 :70, 35:65), 0.8 &1.0ml/min | Fig. 6.19, 6.20, 6.21 &6.22 |
| 3 | ACN: acetate buffer, 30mM, pH 3.0 30 :70), 1.0ml/min | Fig. 6.23 |
| 4 | ACN: acetate buffer, 50mM, pH 3.0 (30 :70), 1.0ml/min | Fig. 6.24 |
| 5 | ACN: 0.1% formic acid (pH not adjusted); 25:75 | Fig. 6.25, 6.26 |
| 6 | ACN: 0.1% TEA, pH adjusted with Formic acid (17.5:82.5) (pH 3.0) | Fig. 6.27 |
| 7 | 15:85 of ACN: 0.1% TEA, pH adjusted to 3.5 with Formic acid | Fig. 6.28 |

The outcomes of the trials are presented in Table 6.64 (pp 218) and corresponding chromatograms shown as Fig.6.16 to Fig.6.28 (pp 219 to 225).

Mobile phase of ACN: 0.1% TEA, adjusted to pH 3.0 with formic acid in ratio of 15:85 at a flow rate of 0.8 mL/min achieved good resolution between LEV and its degradants. The experimental variables established for proposed Method 3 are presented in Table 6.65 (pp 226) and the resulting chromatograms are presented as Fig.6.29 and 6.30 (pp 225 and 226).

### 5.3.3.3 System Suitability and Validation of Method 3

#### 5.3.3.3.1 Retention Time:

Retention time was assessed with six replicate injections of FQs at a concentration of 20 µg/mL prepared as stated in section 5.3.2.1 (pp 137).

#### 5.3.3.3.2 Column Efficiency:

Column efficiency, represented by N, the number of theoretical plates, was assessed by six replicate injections of solutions (standard and stress induced sample) prepared as stated in section 5.3.2.1 (pp 137).

#### 5.3.3.3.3 Peak Symmetry:

Peak symmetry was also assessed by the same process described in section 5.3.2.3 (pp 139).

#### 5.3.2.3.4 Resolution:

Resolution between the drug and degradant peaks was used for evaluating the stability indicating capability of proposed methods.

The results of System Suitability observed for six replicate injections have been presented in Table 6.66 (pp 227).

**Validation Parameters**

**5.3.3.3.5 Precision:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of diluted tablet extract (conc. 50 µg/mL) on two different days. Results of the study are presented in Table 6.67 (pp 228).

**5.3.3.3.6 Linearity:**

Stock solution of 1000 µg/mL concentration was prepared for LEV as per procedure provided in section 5.2.1 (pp 121). Aliquots were taken from stock solution of LEV, as per volumes shown in Table 5.8, and diluted to give standard solutions of 10-120 µg/mL concentration.

**Table 5.8 Preparation of Standard Solutions of LEV for Linearity Studies**

| Volume of LEV from Stock in mL | Final Volume made up to (mL) | Resultant Concentration (µg/mL) |
|---|---|---|
| 0.1 | 10 | 10 |
| 0.2 | 10 | 20 |
| 0.3 | 10 | 30 |
| 0.5 | 10 | 50 |
| 0.8 | 10 | 80 |
| 1.0 | 10 | 100 |
| 1.2 | 10 | 120 |

These resultant solutions were injected into the chromatograph under optimized experimental conditions. The peak area versus concentration relationship was plotted to establish the regression equation and correlation coefficient.

The results of linearity studies for LEV have been tabulated in Table 6.68 (pp 229), Fig. 6.31 (pp 229).

**5.3.2.3.7 Limit of Detection (LOD) and Limit of Quantification (LOQ):**

The method's limit of detection (LOD) and limit of quantification (LOQ) were obtained from the linearity plot.

**5.3.3.3.8 Accuracy**

Spiking technique was used to assess the accuracy of the procedure under consideration. To previously analyzed sample solutions of LEV tablet extract (conc. of 50 µg/mL) were added standard solutions of various concentrations at levels ranging between 80 - 120 percent, as shown in Table 5.3 (pp 129).

The resultant solutions were subjected to chromatographic analysis using the optimised mobile phase. The percentage of concentration recovered and the percentage of RSD at each dose level of LEV were calculated and compared. At each level, the responses were examined with three replicate injections and the results for LEV have been reported in Table 6.69 (pp 230).

**5.3.3.3.9 Robustness**

The robustness of the approach was tested by observing the impact of minor but deliberate changes in experimental variables like the composition of the mobile phase solution, pH

of aqueous component, and flow rate on the performance of the method. Experimental parameters altered were pH of aqueous component in mobile phase (±0.2 units), proportion of mobile phase components (±2%), and flow rate (±0.2 mL/min) of mobile phase. Robustness was evaluated by calculating the percent RSD.

The effect of ±2% variation in the mobile phase proportion, namely the ratio of organic phase to aqueous phase, 13:87 and 17:83 of ACN: 0.1 % TEA as compared to 15:85 used in the proposed optimized method, has been studied and the results presented in Table 6.70 (pp 231), for LEV and stress induced samples containing degradant LDA.

The impact of change in pH of aqueous phase to 2.8 and 3.2 as compared to pH 3.0 used in the optimized method (resulting in ±0.2 units variation) was investigated and has been tabulated in Table 6.71 (pp 231) for LEV and its stress induced sample.

Variation in mobile phase flow rate by working at 0.6 mL/min and 1.0 mL/min as compared to 0.8 mL/min used in the proposed method (±0.2 mL variation) was checked and the outcome has been presented in Tables 6.72 (pp 232) for LEV and its degraded sample.

**5.3.3.3.10 Assay of Marketed Formulation**

The proposed HPLC method was extended for the determination of LEV in tablets (Leon 500 (batch no. LB80304) manufactured by Dr. Reddy's Laboratories Ltd.). It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 3.

The system suitability and validation results of Method 3 are summarised in Table 6.73 (pp 233).

Method 3 for analysis of LEV and degradants has been satisfactorily validated. Trials were also performed to extend this method to analysis of CIP and its degradants.

### 5.3.4 Method 4: Method Development for Analysis of CIP on LC-MS

#### 5.3.4.1 Procedure for Preparation of Solutions for Method 4:

**Preparation of Stock and Working (Standard) Solutions of FQs:**

Stock solution of 1000 µg/mL concentration were prepared for CIP as per procedure provided in section 5.2.1 (pp 121). From the stock solution of CIP, a working solution was prepared resulting in final concentration of 20 µg/mL.

**Preparation of Degraded Samples:**

Solutions of CIP, subjected to stress as per procedures stated in section 5.3.1.5 (pp 132) were used for developing a new stability indicating method.

CDA refers to the degradant produced upon acid hydrolysis of CIP and CDO to the degradant formed in oxidised sample of CIP.

Stressed sample of CIP undergoing acid hydrolysis showed two degradant peaks that were termed as CDA1 and CDA2. The oxidative stress resulted in only one degradant peak termed CDO.

#### 5.3.4.2 Trials towards Development of Method 4

Attempts were made to extend Method 3 earlier developed for LEV to analyse CIP and its possible degradants for the purpose of their identification with Mass Spectrometry (LC-MS).

**Trials:** The method parameters of Method 3 were tried for CIP and its degradants. Some of the trials conducted to find suitable LC-MS compatible HPLC method for CIP and its degradants are presented in Table 5.9.

**Table 5.9. Trials conducted to find suitable LC-MS compatible HPLC method for CIP and its degradants**

| Trial No. | Experimental Conditions (Mobile phase, pH, flow rate) | Chromatogram |
|---|---|---|
| 1 | ACN and 0.1% TEA, pH 3.0 (20:80; 0.8 mL/min) | Fig. 6.32 |
| 2 | ACN and 0.1% TEA, pH 3.0 (15:85; 0.8 mL/min) | Fig. 6.33 |
| 3 | ACN and 0.1% TEA, pH 3.0 (13:87, 0.8 mL/min) | Fig. 6.34 |
| 4 | ACN and 0.1% TEA, pH 3.0 (13:87, 1.0 mL/min) | Fig. 6.35 |
| 5 | ACN and 0.1% TEA, pH 3.0 (13:87, 1.5 mL/min) | Fig. 6.36, 6.37 |

The outcomes of the trials are reported in Table 6.74 (pp 234) and corresponding chromatograms shown in Fig.6.32 to Fig.6.37 (pp 235 to 237).

Method using a mobile phase having components same as in Method 3, namely, ACN and 0.1% TEA adjusted to pH 3.0 with formic acid in a slightly different proportion of 13:87, but at a higher flow rate of 1.5 mL/min to keep the run time shorter, was found to be suitable to CIP and its degradants.

The experimental variables for proposed Method 4 are presented in Table 6.75 (pp 238) and the resulting chromatograms are presented as Fig.6.33 and 6.34 (pp 235, 236).

### 5.3.4.3 System Suitability and Validation of Method 4

#### 5.3.4.3.1 Retention Time:

Retention time was assessed with six replicate injections of degraded sample of CIP at a concentration of 20 µg/mL prepared as stated in section 5.3.2.1.(pp 137)

#### 5.3.4.3.2 Column Efficiency:

Column efficiency represented by the number of theoretical plates (N), was assessed with six replicate injections of solutions (standard and degraded sample) prepared as stated in section 5.3.2.1.(pp 137)

#### 5.3.4.3.3 Peak Symmetry:

Peak symmetry was also assessed by the same process described in section 5.3.2.3(pp 139)

#### 5.3.2.3.4 Resolution:

Resolution between the drug and degradant peaks was used for evaluating the stability indicating capacity of proposed methods.

The results of System Suitability observed for six replicate injections have been presented in Table 6.76 (pp 238).

**Validation Parameters:**

**5.3.4.3.5 Precision:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of diluted tablet extract (conc. 50 µg/mL) on two different days. Results are presented in Table 6.77 (pp 239).

**5.3.4.3.6 Linearity:**

Stock solution of 1000 µg/mL concentration was prepared for CIP as per procedure provided under section 5.2.1 (pp 121). Aliquots were taken from stock solution of CIP, as per volumes shown in Table 5.10, and diluted to give standard solutions of 10-120 µg/mL concentration.

**Table 5.10 Preparation of Standard Solutions of CIP for Linearity Studies**

| Volume of CIP from Stock in mL | Final Volume made up to (mL) | Resultant Concentration (µg/mL) |
|---|---|---|
| 0.1 | 10 | 10 |
| 0.2 | 10 | 20 |
| 0.3 | 10 | 30 |
| 0.5 | 10 | 50 |
| 0.8 | 10 | 80 |
| 1.0 | 10 | 100 |
| 1.2 | 10 | 120 |

These resultant solutions were injected into the chromatographic column under optimal

chromatography conditions. The peak area versus concentration relationship was plotted to determine the regression equation and correlation coefficient. The results of linearity studies for CIP have been reported in Table 6.78 (pp 240), Fig. 6.38 (pp 240).

**5.3.4.3.7 Limit of Detection (LOD) and Limit of Quantification (LOQ):**

The method's limit of detection (LOD) and limit of quantification (LOQ) were obtained from the linearity plot.

**5.3.4.3.8 Accuracy**

Spiking technique was used to assess the accuracy of the procedure under consideration. To previously examined sample solutions of CIP tablet extract dilution (conc. of 50 µg/mL) were added standard solutions of various concentrations at levels ranging between 80 - 120 percent, as shown in Table 5.3 (pp 129)

The resultant solutions were subjected to chromatographic analysis using the optimised mobile phase. The percentage of concentration recovered and the percentage of RSD at each dose level of CIP were calculated and compared. At each level, the responses were examined with three replicate injections and the results for CIP have been reported in Table 6.79 (pp 241).

**5.3.4.3.9 Robustness**

The robustness of the approach was tested by observing the impact of minor but deliberate changes in experimental variables like the composition of the mobile phase solution, pH of aqueous component, and flow rate on the performance of the method. Experimental parameters altered were pH of aqueous component in mobile phase (±0.2 units),

proportion of mobile phase components (±2%), and flow rate (±0.2 mL/min) of mobile phase. Robustness was evaluated by calculating the % RSD.

The effect of ±2% variation in the mobile phase proportion, namely the ratio of organic phase to aqueous phase, 15:85 and 11:89 of ACN: 0.1 % TEA as compared to 13:87 used in the proposed optimized method, has been studied and the results presented in Table 6.80 (pp 242), for CIP and its stress induced samples containing degradants CDA1 and CDA2.

The impact of change in pH of aqueous phase to 2.8 and 3.2 as compared to pH 3.0 used in the optimized method (resulting in ±0.2 units variation) was investigated and has been tabulated in Table 6.81 (pp 242) for CIP and its degraded sample.

Variation in mobile phase flow rate by working at 1.3 mL/min and 1.7 mL/min as compared to 0.8 mL/min used in the proposed method (±0.2 mL variation) was checked and the outcome has been presented in Tables 6.82 (pp 243) for CIP and its degraded sample.

**5.3.4.3.10 Assay of Marketed Formulation**

The proposed HPLC method was extended for the determination of CIP in tablets (Ciplox- 500 (batch no. SB60176) manufactured by Cipla Ltd.). It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 4.

The system suitability and validation results of Method 4 are summarised in Table 6.83 (pp 244).

LC-MS studies were performed on the degraded samples of LEV and CIP using Methods 3 and 4, both of which were LC-MS compatible.

### 5.4 LCMS Studies

The stress degraded samples of CIP and LEV have been subjected to LCMS studies for characterization of components (parent drug and degradants) that have emerged as peaks in the HPLC, using methods 3 & 4.

Liquid Chromatography coupled with Mass Spectrometry is an analytical technique which combines physical separation of components (drug and its degradant, in this case) with identification of separated components eluting as peaks. Soft ionization ESI (electrospray ionization) technique was used for mass fragmentation on the LC-MS 6400 Series Triple Quadrupole B.08.00 (B8023.5 SP1) of Agilent Technologies.

### 5.5 Comparison of Proposed Methods with Existing methods

The newer methods are compared with HPLC methods reported in literature. The data collected has been presented in section 3.4, Review of Literature, Chapter 3 (pp 46).

### 5.6 Prediction of Retention times.

The prediction of analyte retention factor in RP-LC helps largely in method development since it considerably simplifies the optimization procedures to find the best experimental conditions. Several models have been proposed to estimate the chromatographic behavior of the solutes in RP-LC. Some of these approaches are based on solute–mobile phase interactions, where descriptors such as the organic modifier volume fraction in the mobile phase and mobile phase polarity are used. However accurate predictions could be done only in a specific range of mobile phase compositions. In order to overcome this limitation, more complete models have been introduced to try and explain the chromatographic behavior using mobile phase–solute–stationary phase interactions. The

scope of identifying an appropriate model for prediction of Rt has been explored in the current study.

The retention in reversed-phase liquid chromatography has been related to solute, mobile phase and stationary phase relative polarity parameters [364–367].

### 5.6.1. Collection of data on existing HPLC methods for multiple FQs

In the work presented here published HPLC methods for estimation of multiple fluoroquinolones were referred to and attempts were made to establish relation between solute descriptors and retention time.

### 5.6.2. Calculation of Physicochemical properties of fluoroquinolones

Physicochemical properties of fluoroquinolones were calculated using OSIRIS DataWarrior and Chemaxon Software.

The universal data analysis and visualisation tool DataWarrior is a flexible tool for examining huge data sets of chemical compounds with alphanumerical attributes thanks to its incorporated cheminformatics algorithms[368,369]. To assist synthetic and medicinal chemists in their daily work, DataWarrior uses both old and new cheminformatics algorithms. In order to assist chemists in making wiser judgements about structural alterations toward better property profiles, these techniques include combinatorial library enumeration, the prediction of molecular characteristics, and numerous approaches to display chemical space and activity cliffs. To visualise the chemical space of medium-sized compound collections, the new 2-dimensional scaling technique "Rubber Band Scaling" was introduced and contrasted with principal component analysis and the self-organizing map. The approach has the benefit that no

molecules are concealed behind others and that all molecule markers are uniformly distributed. Therefore, this strategy works well when all molecules must be displayed, such as when illustrating activity space or activity cliffs. This method, in contrast to PCAs and SOMs, may be applied to complicated, non-vector descriptors like the Flexophore, which makes it possible to view the probable binding behavior space of chemical libraries. The DataWarrior application includes the Rubber Band Scaling method.

### 5.6.3. Application of Multiple Linear Regression analysis

Multiple Linear Regression analysis was applied to each selected method separately to determine the most relevant solute descriptors.

Applicability of certain Molecular Descriptors was studied to investigate their effect on Retention Time. These are:

1. cLogP (Octanol-water partition coefficient; partitioning): *The octanol/water partition coefficient ($K_{ow}$) is defined as the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase of a two-phase octanol/water system*[370–372].
2. LogS (water solubility): *It is defined as a common solubility unit corresponding to the 10-based logarithm of the solubility of a molecule measured in mol/L*[373].
3. Topological Surface Area (TSA, affects binding to stationary phase, ease of movement): *The polar surface area (PSA) or topological polar surface area (TPSA) of a molecule is defined as the surface sum over all polar atoms or molecules, primarily oxygen and nitrogen, also including their attached hydrogen atoms*[374].

4. Relative Polar Surface Area (Rel PSA, SAS, surface area of polar groups): *It is defined as the surface area of the molecule accessible to solvent*[374].
5. LogD (at pH used in method, affects ionisation): *LogD is the ratio of the sum of the concentrations of all species of the compound in octanol to the sum of the concentrations of all species of the compound in water*[372].

The methods involving gradient elution were not included in the study as that could lead to difficulty with prediction of retention time/ factor with a continuous change in composition of mobile phase proportion. Also, optimized methods involving less than four FQs were omitted as statistical calculations involving multiple linear regression need sample size of minimum 4 for studying effect of at least 2 variables (X) on retention time/ factor (Y).

Attempts were made at establishing correlation between these Molecular Descriptors/ physicochemical properties and Retention Time by using Multiple Linear Regression.

### 5.6.4. Evaluation & selection of Multiple Linear Regression

The models or regression equations thus generated were evaluated for their prediction capability using F-test and $R^2$.

An F test is a statistical test that has an F-distribution under the null hypothesis. It is used to compare statistical models according to the provided data set[375].

Here the null hypothesis along with the alternate hypothesis. The F-value is calculated, using the formula. This F-statistic formula is the ratio of the variance of the group means divided by the mean of the within-group variances. The F Statistic which is the critical value for this test is determined and, on this basis, the Null Hypothesis is accepted or rejected.

$R^2$ is a metric used in statistics to determine how well a regression line matches actual data. R squared calculates the degree of difference between the regression model's predicted and observed values.

### 5.6.5. Calculation of Physicochemical properties of FQ related substances

Physicochemical properties of related substances of LEV were calculated using OSIRIS DataWarrior and Chemaxon Software.

### 5.6.6 Prediction of Retention Times for Related Substances of LEV

One of the selected HPLC methods (Method 10) was further investigated and those models which showed promise were further studied for their prediction capacity by using reported degradants / related substances of LEV.

### 5.6.7 Application of ANOVA to Predicted Retention Times

Analysis of variance (ANOVA) is an analysis tool used in statistics. One-way (or unidirectional) and two-way ANOVA are the two main varieties. ANOVA also comes in several forms[376]. For instance, MANOVA (multivariate ANOVA) is different from ANOVA in that the former evaluates several dependent variables simultaneously while the latter only does so for one. The number of independent variables in your analysis of variance test—one or two—is referred to as one-way or two-way. To evaluate if there are any statistically significant differences between the means of three or more independent (unrelated) groups, the one-way ANOVA is utilised. The one-way ANOVA is expanded upon by the two-way ANOVA. One independent variable influences one or more dependent variables in a one-way relationship. A two-way ANOVA has two independent variables. It is used to examine the simultaneous effects of two factors and observe how the two elements interact.

ANOVA procedure involves calculating all the means for all the groups. The null hypothesis assumes that there is no variance data in different groups. The alternate hypothesis states the means are different. The sum of squares (SS) based on the entire set in all the groups is calculated.

$$SS_{total} = \sum_{j=1}^{n} (\bar{X}_j - \bar{X})^2$$

Then the total degree of freedom (DFT), Degrees of Freedom Within Groups (DFW) and Degrees Between Groups (DFB) are calculated using following formulae:

DFT= n- 1

Where, n is to total of all the data sets combined.

DFW= k- 1

Where, k is the number of groups.

DFB= n- k

The mean square between (MSB) and mean square within (MSW) is calculated by using following formulae:

$$MS_{between} = \frac{SS_{between}}{df_{between}}$$

$$MS_{w\ ithin} = \frac{SS_{within}}{df_{within}}$$

The F statistic is then calculated using the formula:

$$F = \frac{MS_{between}}{MS_{within}}$$

The calculated F values (absolute value) are compared with the tabulated F values. If the absolute value is greater than the critical value, we reject the null hypothesis and conclude that there is significant different between the means of the populations. Otherwise, we accept the null hypothesis and conclude that there is no significant difference.

Statistical test ANOVA was applied to the predicted Retention Times of each of the related substances reported for LEV.

# *CHAPTER 6*

## *RESULTS AND DISCUSSION*

## 6. Results and Discussion

### 6.1 Exploratory Trials: Application of existing methods to other FQs.

System Suitability Parameters for FQs of research interest using mobile phase Methanol: 0.1% orthophosphoric acid (OPA) in the ratio 40:60, at a flow rate of 2 mL/min, and elevated temperature of 50 °C have been tabulated in Table 6.1.

**Table 6.1. System Suitability Parameters for some FQs using mobile phase Methanol: 0.1% orthophosphoric acid (OPA) in the ratio 40:60, at a flow rate of 2 mL/min, and elevated temperature of 50°C**

| Sr. No. | Drug | Rt (min) | N | Peak Symmetry |
|---|---|---|---|---|
| 1 | CIP | 4.78 | 1764 | 0.858 |
| 2 | LEV | 4.98 | 1089 | 1.039 |
| 3 | MOX | 10.76 | 1421 | 1.007 |
| 4 | NOR | 18.54 | 5329 | 0.898 |
| 5 | OFL | 4.93 | 1069 | 1.070 |

(Acceptance Criteria: N ≥ 2000 and peak symmetry 0.8 to 1.2)

It was observed that the method when applied to other FQs, namely, CIP, MOX, LEV and OFL, provided results that were not satisfactory with respect to system suitability. Modification of organic phase ratio, flow rate, pH with the objective of improving the system suitability parameters and arriving at a method most suitable for analysis of FQs was considered.

Among the various trials carried out, the method showing improved outcomes was observed with mobile phase comprising of Methanol: 0.1% OPA (70:30), at flow rate of

1 mL/min, 40°C column temperature, has been reported in Table 6.2. This method showed high tailing and low N for LEV and OFL as is visible from data summarised.

**Table 6.2. System Suitability Parameters for FQs using mobile phase with Methanol: 0.1%OPA in the ratio 70:30, at flow rate 1 mL/min, and elevated temperature of 40 °C**

| Sr. No. | Drug | Rt (min) | N | Peak Symmetry |
|---|---|---|---|---|
| 1 | CIP | 4.22 | 4318 | 1.592 |
| 2 | LEV | 4.97 | 2000 | 1.939 |
| 3 | MOX | 4.97 | 2517 | 1.658 |
| 4 | NOR | 5.48 | 5556 | 0.863 |
| 5 | OFL | 4.96 | 1932 | 1.955 |

It was generally observed that the compendial methods varied for each of the FQs and could not be applied to other FQs in spite of structural similarities due to system suitability data not being within the acceptance criteria.

These unsuccessful attempts necessitated development of appropriate analytical method that could be applied to several FQs and their marketed dosage forms.

## 6.2 Method Development and Validation.

Experimental parameters such as flow rate, pH along with change in proportion of organic phase and buffer were explored and system suitability factors used to evaluate results.

The effect of mobile phase composition and flow rate on retention time and peak symmetry has been reported in Table 6.3 A & B.

**Table 6.3A. Effect of mobile phase composition (Methanol: Phosphate buffer pH 2.7) and flow rate on retention time and peak symmetry**

| Drug | Flow rate | 1.25 mL/min |
|---|---|---|
| | Composition | 70:30 |
| Ciprofloxacin | Rt (min) | 2.092 |
| | Tailing | 0.886 |
| | N | 1856 |
| Levofloxacin | Rt(min) | 2.083 |
| | Tailing | 0.884 |
| | N | 1949 |
| Moxifloxacin | Rt(min) | 2.167 |
| | Tailing | 0.846 |
| | N | 2270 |
| Ofloxacin | Rt(min) | 2.083 |
| | Tailing | 0.876 |
| | N | 2007 |
| Norfloxacin | Rt(min) | 2.817 |
| | Tailing | 1.181 |
| | N | 3906 |

**Table 6.3B. Effect of mobile phase composition (Methanol: Phosphate buffer pH 2.7) and flow rate on retention time and peak symmetry.**

| Drug | Flow rate | 1.0 mL/min | 1.25 mL/min | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ratio | 70:30 | 70:30 | 65:35 | 60:40 | 55:45 | 50:50 | 40:60 |
| Levofloxacin | Rt(min) | 2.608 | 2.083 | 2.108 | 2.125 | 2.175 | 2.308 | 2.908 |
| | Tailing | 0.831 | 0.884 | 0.895 | 0.835 | 0.770 | 0.748 | 0.730 |
| | N | 2870 | 1949 | 1873 | 1653 | 1299 | 903 | 451 |
| Moxifloxacin | Rt(min) | 2.708 | 2.167 | 2.233 | 2.352 | ____ | 3.000 | 5.75 |
| | Tailing | Bad shape | 0.846 | 0.801 | 0.842 | ____ | 0.862 | 0.672 |
| | N | 2570 | 2270 | 1792 | 1396 | ____ | 817 | 699 |

Mobile phase composition with ratio of 70:30 Methanol: Phosphate buffer pH 2.7 at 1.25 mL/min was found to give promising results since the peak symmetry was found to be good for all five drugs of research interest. Number of theoretical plates (N) was also found to be satisfactory in most cases. Further trials for optimizing mobile phase compositions were carried out at flow rate 1.25 mL/min.

**Table 6.4. Effect of mobile phase (Methanol: Phosphate buffer pH 2.7) and flow rate**

| Drug | Flow rate | 1.25 mL/min | | |
|---|---|---|---|---|
| | Composition | 70:30 | 65:35 | 60:40 |
| Levofloxacin | Rt(min) | 2.083 | 2.092 | 2.108 |
| | Tailing | 0.942 | 0.897 | 0.850 |
| | N | 2007 | 2043 | 1674 |
| Moxifloxacin | Rt(min) | 2.167 | 2.217 | 2.325 |
| | Tailing | 0.835 | 0.846 | 0.893 |
| | N | 2368 | 1867 | 1410 |
| Ofloxacin | Rt(min) | 2.083 | 2.083 | 2.108 |
| | Tailing | 0.865 | 0.833 | 0.829 |
| | N | 2191 | 2040 | 1706 |
| Ciprofloxacin | Rt(min) | 2.092 | 2.108 | 2.15 |
| | Tailing | 0.884 | 0.837 | 0.819 |
| | N | 2013 | 1938 | 1596 |
| Norfloxacin | Rt(min) | 2.808 | 3.167 | 3.717 |
| | Tailing | 1.196 | 1.113 | 1.079 |
| | N | 3928 | 3712 | 3589 |

From literature fluoroquinolones generally were shown to possess two pKa values, one at around 5 and other at 8. Silica columns are generally considered to be damaged at pH > 8, trials were continued with pH of mobile phase in the mid-acidic region of 2.5 to 3.5.

**Table 6.5. Effect of pH of mobile phase Methanol: Phosphate buffer (70:30) at 1.25 mL/min flow rate**

| Drug ↓ | Flow rate→ Composition→ | 1.25 mL/min 70:30 | | |
|---|---|---|---|---|
| | pH→ | 2.7 | 3.0 | 3.3 |
| Levofloxacin | Rt(min) | 2.083 | 2.108 | 2.125 |
| | Tailing | 0.942 | 0.867 | 0.912 |
| | N | 2007 | 3801 | 1820 |
| Moxifloxacin | Rt(min) | 2.167 | 2.158 | 2.167 |
| | Tailing | 0.835 | 0.882 | 0.883 |
| | N | 2368 | 2505 | 2669 |
| Ofloxacin | Rt(min) | 2.083 | 2.108 | 2.117 |
| | Tailing | 0.865 | 0.872 | 0.943 |
| | N | 2191 | 3888 | 1909 |
| Ciprofloxacin | Rt(min) | 2.092 | 2.108 | 2.108 |
| | Tailing | 0.884 | 1.673 | 1.770 |
| | N | 2013 | 4696 | 4566 |
| Norfloxacin | Rt(min) | 2.808 | 2.800 | 2.792 |
| | Tailing | 1.196 | 1.167 | 1.200 |
| | N | 3928 | 3913 | 3888 |

Considering the Number of Theoretical Plates (N) and shape of peaks in chromatograms, pH 3.0 was found to be most suitable. Further trials were carried out to finalise the composition of mobile phase by varying the proportion of Phosphate buffer pH 3.0, at flow rate 1.25 mL/min

**Table 6.6. Effect of mobile phase Methanol: Phosphate buffer (adjusted to pH 3.0), at 1.25 mL/min flow rate**

| Drug | Composition→ | 70:30 | 65:35 | 60:40 |
|---|---|---|---|---|
| Levofloxacin | Rt(min) | 2.100 | 2.1000 | 2.125 |
| | Tailing | 0.911 | 0.888 | 0.829 |
| | N | 3934 | 3253 | 2322 |
| Moxifloxacin | Rt(min) | 2.158 | 2.208 | 2.317 |
| | Tailing | 0.916 | 0.869 | 0.879 |
| | N | 2430 | 1919 | 1465 |
| Ofloxacin | Rt(min) | 2.100 | 2.100 | 2.117 |
| | Tailing | 0.961 | 0.892 | 0.880 |
| | N | 4022 | 3320 | 2375 |
| Ciprofloxacin | Rt(min) | 2.100 | 2.108 | 2.150 |
| | Tailing | 0.903 | 0.883 | 0.836 |
| | N | 2660 | 2273 | 1792 |
| Norfloxacin | Rt(min) | 2.792 | 2.150 | 3.642 |
| | Tailing | 1.191 | 0.836 | 1.180 |
| | N | 3936 | 1792 | 3715 |

It was generally observed from data captured there was a decrease in Number of Theoretical plates (N) with reduction in the proportion of methanol in the mobile phase and also peak shape was not satisfactory (showing visible distortion, and increased peak width, thereby decreasing N).

**Effect of Column Temperature:**

The column in the instrument used was installed in a column oven, wherein the temperature can be programmed from 0-100 °C. The effect of column temperature on system suitability parameters was studied from ambient up to 60 °C.

**Table 6.7. Effect of Column Temperature**

| Temperature→ | 30 °C | 40 °C | 50 °C | 60 °C |
|---|---|---|---|---|
| Rt(min) | 2.11 | 2.12 | 2.11 | 2.10 |
| Tailing | 1.03 | 1.01 | 1.00 | 0.99 |
| N | 3951.00 | 4132.67 | 4176.00 | 4282.67 |
| Peak area | 1462404.00 | 1452108.00 | 1437611.00 | 1456193.00 |
| Peak height | 234281.30 | 233872.00 | 232927.00 | 237756.70 |

Since there were no marked differences observed in system suitability data with changes in temperature, it was decided to select 40 °C as temperature for column oven as this was the default temperature set for the instrument.

**Effect of loop volume:**

Loop volumes of different magnitude were considered while optimization to verify impact of loop volume on system suitability data.

**Table 6.8. Effect of Loop Volume**

| Drug | Loop Volume→ | 10 µl | 20 µl |
|---|---|---|---|
| Levofloxacin | Rt(min) | 2.075 | 2.075 |
| | Tailing | 1.148 | 1.000 |
| | N | 3302 | 2819 |
| | Peak area | 393350 | 764619 |
| | Peak height | 63780 | 109700 |
| Moxifloxacin | Rt(min) | 2.142 | 2.133 |
| | Tailing | 1.265 | 1.011 |
| | N | 3529 | 3293 |
| | Peak area | 802806 | 893076 |
| | Peak height | 135242 | 142107 |
| Ofloxacin | Rt(min) | 2.075 | 2.067 |
| | Tailing | 1.158 | 0.892 |
| | N | 3404 | 3320 |
| | Peak area | 433569 | 871389 |
| | Peak height | 72938 | 127365 |
| Ciprofloxacin | Rt(min) | 2.075 | 2.075 |
| | Tailing | 1.055 | 0.920 |
| | N | 3096 | 2229 |
| | Peak area | 178413 | 352503 |
| | Peak height | 27685 | 45353 |
| Norfloxacin | Rt(min) | 2.758 | 2.742 |
| | Tailing | 1.229 | 1.234 |
| | N | 5723 | 3744 |
| | Peak area | 120385 | 248095 |
| | Peak height | 20801 | 35676 |

Besides the obvious increase in peak area and peak height with increase in loop volume, a decrease in Number of Theoretical Plates was observed. However, the Number of Theoretical plates was found to remain well within acceptance limits. The peak symmetry or tailing was optimum with loop volume of 20 µL, hence considered for validation studies.

UV absorption spectra were recorded for all 5 FQs of research interest. In the matter of CIP at 294 nm the absorptivity was 45% of maxima; also, absorptivity was slightly less (459) to that of few others. It was proposed as detection wavelength to harmonise experimental for a group of drugs belonging to similar chemical class without compromising on sensitivity of detection. The UV spectra of FQs selected for study have been presented as Fig. 6.A.

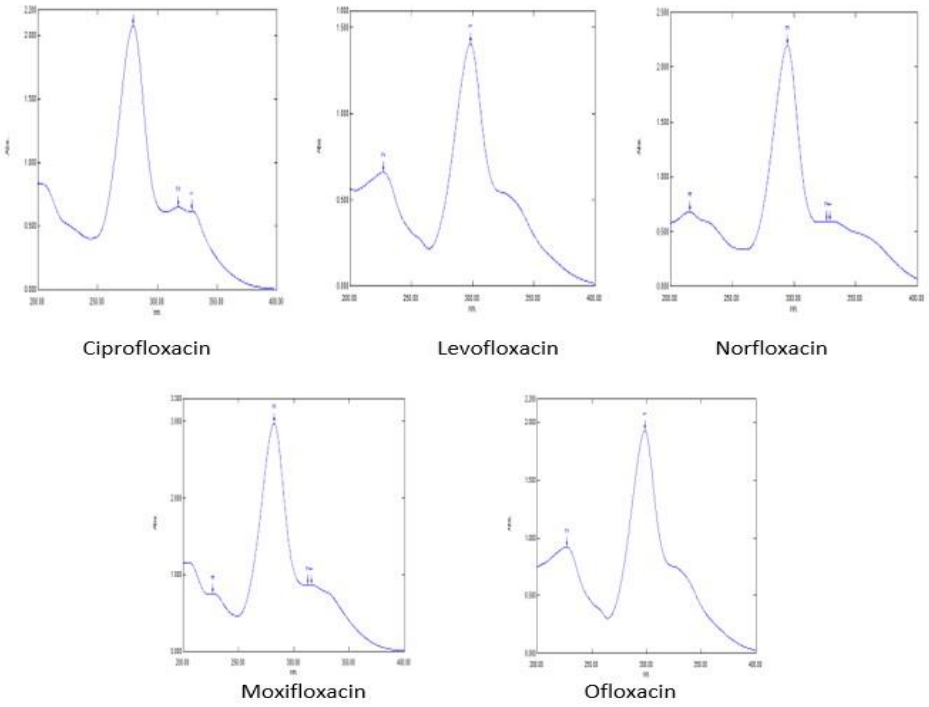



**Fig.6.A. UV Absorption Spectra of FQs**

### 6.2.1 Method 1

From the trials, it was observed that Methanol: Phosphate buffer pH 3.0 in the proportion 70:30 showed best results to meet acceptance criteria (Table 6.10B) wherein flow rate of 1.25 mL/min, detection wavelength of 294 nm and column temperature of 40 °C was used.

Method 1 was subjected to optimization and validated for analysis of FQs of research interest. Results of study are presented in Table 6.9.

**Table 6.9. Developed Method 1**

| Method No. | Column | Column Temperature | Mobile Phase | Flow rate (mL/min) | Applicable to |
|---|---|---|---|---|---|
| 1 | C18 | 40 °C | Methanol: Phosphate buffer pH 3.0, (70:30) in isocratic mode | 1.25 | All selected FQs (CIP, LEV, MOX, NOR, OFL) |

Retention times of FQs were found to be reasonable. Method was validated in accordance with ICH Q2R1 guidelines and system suitability data found to be satisfactory. Representative chromatograms are presented as Fig. 6.1, 6.2 and 6.3.

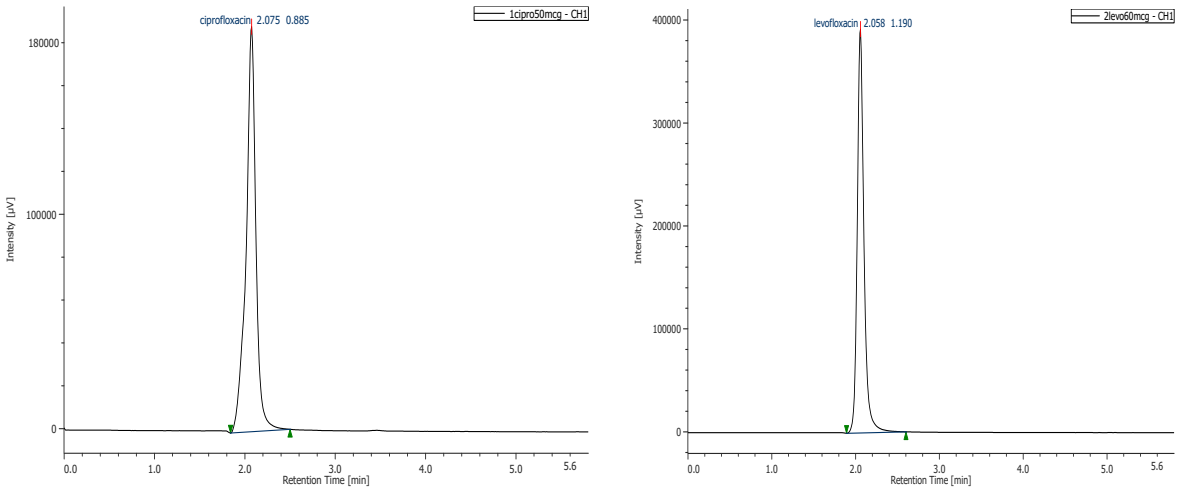



**Fig.6.1. Chromatograms of CIP and LEV using mobile phase Methanol: phosphate buffer pH 3.0 (70:30), flow 1.25 mL/min, column temperature 40 °C**

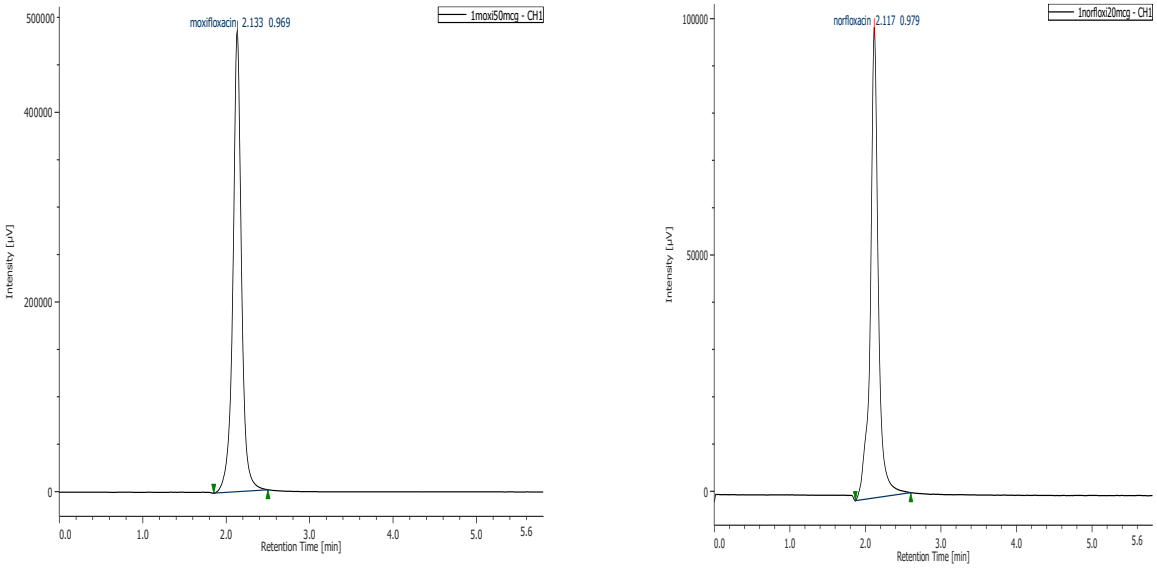



**Fig.6.2. Chromatograms of MOX and NOR using mobile phase Methanol: phosphate buffer pH 3.0 (70:30), flow 1.25 mL/min, column temperature 40 °C**

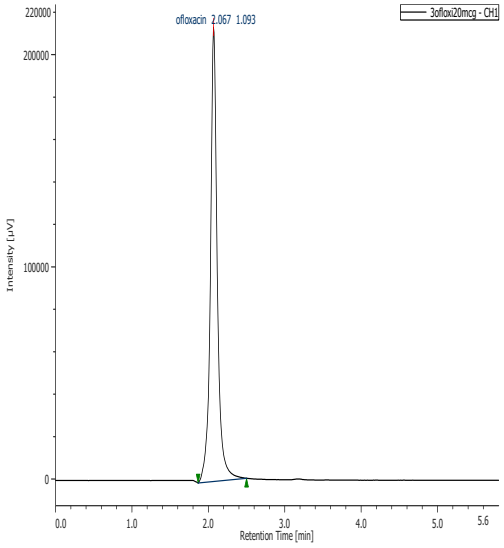



**Fig. 6.3. Chromatogram of OFL using mobile phase Methanol: phosphate buffer pH 3.0 (70:30), flow 1.25 mL/min, column temperature 40 °C**

### 6.2.1.1. System Suitability and Validation of Method 1

#### 6.2.1.1.1 Ciprofloxacin

**Table 6.10A. System suitability of Method 1 for CIP (at conc. 20 µg/mL)**

| Sr. No. | Rt (min) | Peak Area | N | Tailing Factor |
|---|---|---|---|---|
| 1 | 2.100 | 18975 | 2394 | 0.868 |
| 2 | 2.100 | 19074 | 2337 | 0.867 |
| 3 | 2.092 | 18904 | 2408 | 0.900 |
| 4 | 2.092 | 19134 | 2256 | 0.908 |
| 5 | 2.092 | 19347 | 2287 | 0.900 |
| 6 | 2.092 | 19369 | 2282 | 0.904 |
| Mean | 2.095 | 19133.83 | 2327.333 | 0.891 |
| SD | 0.004 | 190.984 | | |
| RSD(%) | 0.197 | 0.998 | | |

**Table 6.10B. System suitability of Method 1 for CIP**

| Sr No | Parameters | Acceptance Criteria | CIP |
|---|---|---|---|
| 1 | Theoretical Plates | >2000 | 2327 |
| 2 | Tailing factor | <2 | 0.891 |
| 3 | RSD of area | <2% | 0.998 |
| 4 | RSD of Ret.Time | <1% | 0.197 |

**Linearity:** The response was linear over concentration range 10-60 µg/ml with $R^2$ value of 0.999. Linearity graph is presented as Fig. 6.4.

**Table 6.11. Linearity data for CIP: Peak areas for Concentration range**

| Conc(µg/ml) | Peak Area* |
|---|---|
| 10 | 11255.33 |
| 20 | 18676.33 |
| 30 | 27390.67 |
| 40 | 36743.67 |
| 50 | 44581.67 |
| 60 | 53492.00 |

*Average of three injections

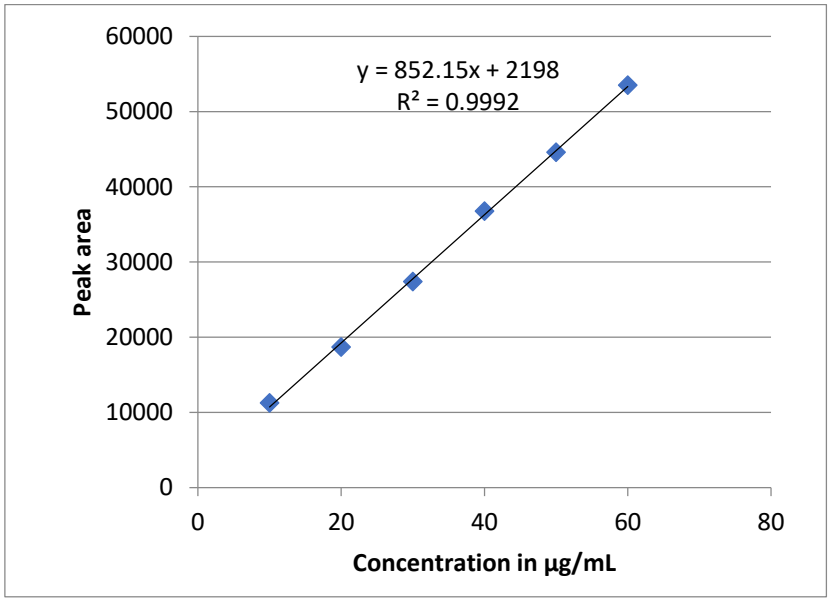



**Fig. 6.4. Linearity Graph for CIP (Method 1)**

Limit of Detection and Limit of Quantitation were calculated and found to be:

Limit of Detection, LOD (calculated) =1.79 µg/ml

Limit of Quantitation, LOQ (calculated) =5.96 µg/ml

**Precision Study of Analysis of CIP by Method 1:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of the tablet extract on two different days.

Acceptance criteria: % RSD should be <2%.

Concentration calculated from the linearity graphs and equations obtained on the respective days. Fresh solutions were prepared each day and intraday precision calculated.

**Table 6.12. Inter- and intraday Precision for CIP (Conc. 20 µg/mL)**

| Day1 | | | Day2 | | |
|---|---|---|---|---|---|
| Sr. No. | Peak Area | Conc(µg/ml) | Sr. No. | Peak Area | Conc(µg/ml) |
| 1 | 18975 | 19.688 | 1 | 19256 | 20.018 |
| 2 | 19074 | 19.804 | 2 | 19274 | 20.039 |
| 3 | 18904 | 19.605 | 3 | 19197 | 19.948 |
| 4 | 19134 | 19.874 | 4 | 19186 | 19.935 |
| 5 | 19347 | 20.124 | 5 | 19006 | 19.724 |
| 6 | 19369 | 20.150 | 6 | 19157 | 19.901 |
| Mean | 19133.83 | 19.874 | Mean | 19179.33 | 19.928 |
| SD | 190.984 | 0.224 | SD | 95.64448 | 0.112 |
| RSD(%) | 0.998 | 1.128 | RSD(%) | 0.498685 | 0.563 |
| Inter-day Precision | | | | | |
| Mean | 19156.58 | 19.901 | | | |
| SD | 145.953 | 0.171 | | | |
| RSD(%) | 0.762 | 0.861 | | | |

**Accuracy:**

Accuracy (% Recovery) was assessed in triplicate, at three different levels equivalent to 80, 100 and 120% of the target concentration of active ingredient, by spiking a known amount of the standard to a sample of known concentration (test solution) and then calculating the recovery and RSD (%) for each concentration as per procedure given in section 5.3.1.4.7 (pp 124).

**Table 6.13. Results of Accuracy of Method 1 for CIP**

| Level of addition (std) (%) | Amount of std added (µg/mL) | Avg. Peak Area | Conc (µg/mL) | Assay | Recovery | % Recovery |
|---|---|---|---|---|---|---|
| 80 | 20 | 43410 | 48.35 | 28.13 | 20.22 | 101.10 |
| 100 | 25 | 47866 | 53.41 | 28.13 | 25.28 | 101.11 |
| 120 | 30 | 52866 | 58.60 | 28.13 | 30.47 | 101.56 |

**Robustness**: The effect of small but deliberate changes in mobile phase proportion, pH and flow rate on retention time, peak area, number of theoretical plates and peak symmetry was studied.

**Effect of mobile phase proportion** (72:28 and 68:32 of methanol: buffer as compared to optimized ratio 70:30 used in the method)

**Table 6.14. Robustness: Effect of Mobile Phase Ratio (for CIP 20 µg/mL)**

| Mobile Phase Ratio | 72:28 | 70:30 (optimized) | 68:32 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 19896.67 | 19940.67 | 20236 | 0.986 |
| %RSD | 0.866 | 0.321 | 0.700 | |

**Effect of variation in pH**: pH of buffer was adjusted to 2.8 and 3.2 as compared to optimized pH of 3.0 used in the method (0.2 units variation)

**Table 6.15. Robustness: Effect of pH (for CIP 20 µg/mL)**

| pH | 2.8 | 3.0 (optimized) | 3.2 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 19918.33 | 19940.67 | 19988.33 | 0.245 |
| %RSD | 0.147 | 0.321 | 0.137 | |

**Effect of variation in mobile phase flow rate**: Flow rate of mobile phase was changed to 1.05 mL/min and 1.45 mL/min as compared to optimized flow rate of 1.25 mL/min used in the method (0.2 mL variation).

**Table 6.16. Robustness: Effect of Mobile Phase Flow Rate (for CIP 20 µg/mL)**

| Mobile Phase Flow Rate (mL/min) | 1.05 | 1.25 (optimized) | 1.45 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 19505.33 | 19940.67 | 20125.33 | 1.385 |
| %RSD | 0.906 | 0.321 | 0.145 | |

**Assay of Marketed Formulation:**

The proposed HPLC method was extended for the determination of CIP in tablets. The tablet used was Ciplox- 500 (batch no. SB60176) manufactured by Cipla Ltd. It was

analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using the proposed Method 1. Sample concentrations 25 µg/mL were injected into the chromatograph and resulting peak area from chromatogram recorded.

Average area (6 injections) for assay solution was calculated to be 815841.

The concentration of sample CIP was calculated to be 25.410 µg/mL and assay results found to be complying 100.05% with label claim (I.P. limits 90-110%).

**6.2.1.1.2 Levofloxacin**

**Table 6.17A. System suitability of Method 1 for LEV (Concentration 20 µg/mL)**

| Sr. No. | Rt (min) | Peak Area | N | Tailing Factor |
|---|---|---|---|---|
| 1 | 2.100 | 1561473 | 3968 | 0.878 |
| 2 | 2.100 | 1556514 | 3778 | 0.896 |
| 3 | 2.092 | 1557721 | 3697 | 0.898 |
| 4 | 2.092 | 1560998 | 3702 | 0.899 |
| 5 | 2.092 | 1559812 | 3779 | 0.882 |
| 6 | 2.092 | 1559763 | 3831 | 0.882 |
| Mean | 2.095 | 1559380 | 3792.5 | 0.889 |
| SD | 0.004 | 1913.015 | | |
| RSD(%) | 0.197 | 0.123 | | |

**Table 6.17B. System suitability of Method 1 for LEV**

| Sr No | Parameters | Acceptance Criteria | LEV |
|---|---|---|---|
| 1 | Theoretical Plates | >1000 | 3792.5 |
| 2 | Tailing factor | <2 | 0.889 |
| 3 | RSD of area | <2% | 0.123 |
| 4 | RSD of Ret. Time | <1% | 0.197% |

**Linearity:** The response was linear from 10-60 µg/ml with a $R^2$ value of 0.999. Linearity graph is seen as Fig. 6.5.

**Table 6.18. Linearity data for LEV: Peak areas for Concentration range**

*Average

| Conc(µg/ml) | Peak Area* |
|---|---|
| 10 | 786636 |
| 20 | 1558786 |
| 30 | 2246531 |
| 40 | 2962968 |
| 50 | 3618195 |
| 60 | 4295056 |

of three injections

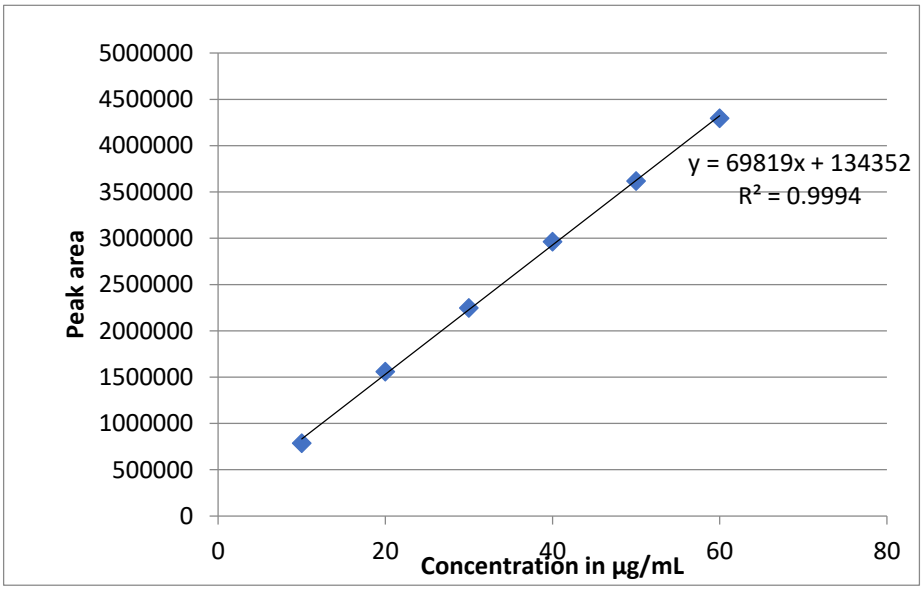



**Fig. 6.5. Linearity Graph for LEV (Method 1)**

Limit of Detection and Limit of Quantitation were calculated and found to be:

Limit of Detection, LOD (calculated) =1.57 µg/ml

Limit of Quantitation, LOQ (calculated) =5.24 µg/ml

**Precision Study of Analysis of LEV by Method 1:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of the tablet extract on two different days.

Acceptance criteria: % RSD should be <2%.

Concentration was calculated from the linearity graphs and equations obtained on the respective days. Fresh solutions were prepared each day and intraday precision calculated.

**Table 6.19. Inter- and intraday Precision for LEV (Conc. 20 µg/mL)**

| Day1 | | | Day2 | | |
|---|---|---|---|---|---|
| Sr. No. | Peak Area | Conc(µg/ml) | Sr. No. | Peak Area | Conc(µg/ml) |
| 1 | 1561473 | 20.440 | 1 | 1557893 | 20.389 |
| 2 | 1556514 | 20.369 | 2 | 1567182 | 20.522 |
| 3 | 1557721 | 20.386 | 3 | 1582334 | 20.739 |
| 4 | 1560998 | 20.434 | 4 | 1561675 | 20.443 |
| 5 | 1559812 | 20.417 | 5 | 1562366 | 20.453 |
| 6 | 1559763 | 20.416 | 6 | 1560993 | 20.433 |
| Mean | 1559380 | 20.410 | Mean | 1565407 | 20.497 |
| SD | 1913.015 | 0.027 | SD | 8818.084 | 0.126 |
| RSD(%) | 0.123 | 0.134 | RSD(%) | 0.563 | 0.616 |
| Inter-day Precision | | | | | |
| Mean | 1564601 | 20.485 | | | |
| SD | 8327.652 | 0.119 | | | |
| RSD(%) | 0.532 | 0.582 | | | |

**Accuracy:**

Accuracy (% Recovery) was evaluated in triplicate, at three levels of concentrations equivalent to 80, 100 and 120% of the target concentration of active ingredient, by spiking

a known amount of the standard to a sample of known concentration and then calculating the recovery and RSD (%) for each concentration as per procedure given in section 5.3.1.4.7 (pp 129).

**Table 6.20. Results of Accuracy of Method 1 for LEV**

| Level of addition (std) (%) | Amount of std added (µg/ml) | Avg. Peak Area | Conc (µg/ml) | Assay | Recovery | % recovery |
|---|---|---|---|---|---|---|
| 80 | 20 | 3357030 | 46.232 | 25.97 | 20.262 | 101.31 |
| 100 | 25 | 3637342 | 50.104 | 25.97 | 24.134 | 96.536 |
| 120 | 30 | 3949088 | 54.670 | 25.97 | 28.700 | 95.667 |

**Robustness**: The effect of changes in mobile phase proportion, pH and flow rate on retention time, peak area, number of theoretical plates and peak symmetry was studied.

**Effect of mobile phase proportion** (72:28 and 68:32 of methanol: buffer as compared to optimized ratio 70:30 used in the method)

**Table 6.21. Robustness: Effect of Mobile Phase Ratio (for LEV 20 µg/mL)**

| Mobile Phase Ratio | 72:28 | 70:30 (optimized) | 68:32 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 1558878 | 1558569 | 1554133 | 0.334 |
| %RSD | 0.153 | 0.166 | 0.570 | |

**Effect of variation in pH**: pH of buffer was adjusted to 2.8 and 3.2 as compared to pH 3.0 optimized for the method (0.2 units variation)

**Table 6.22. Robustness: Effect of pH (for LEV 20 µg/mL)**

| pH | 2.8 | 3.0 (optimized) | 3.2 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 1555432 | 1558569 | 1565128 | 0.299 |
| %RSD | 0.326 | 0.166 | 0.251 | |

**Effect of variation in mobile phase flow rate**: Flow rate of mobile phase was changed to 1.05 mL/min and 1.45 mL/min as compared to optimized 1.25 mL/min used for the method (0.2 mL variation).

**Table 6.23. Robustness: Effect of Mobile Phase Flow Rate (for LEV 20 µg/mL)**

| Mobile Phase Flow Rate (mL/min) | 1.05 | 1.25 (optimized) | 1.45 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 1556977 | 1558569 | 1557523 | 0.328 |
| %RSD | 0.506 | 0.166 | 0.372 | |

**Assay of Marketed Formulation:**

The proposed HPLC method was extended for the determination of LEV in tablets. The tablet used was Leon 500 (batch no. LB60710) manufactured by Dr. Reddy's Laboratories Ltd. It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 1. Sample concentration of 25 µg/mL was injected into the chromatograph and resulting peak area from chromatogram recorded.

Average area (6 injections) for assay solution was calculated to be 1826948.

The concentration of sample LEV sample was calculated to be 25.970 µg/mL and assay results found to be complying 104.19% with label claim (I.P. limits 90-110%).

### 6.2.1.1.3 Moxifloxacin

**Table 6.24A. System suitability of Method 1 for MOX (conc 20 µg/mL)**

| Sr. No. | Rt (min) | Peak Area | N | Tailing Factor |
|---|---|---|---|---|
| 1 | 2.158 | 1420471 | 2395 | 0.867 |
| 2 | 2.150 | 1428320 | 2370 | 0.866 |
| 3 | 2.158 | 1410939 | 2390 | 0.863 |
| 4 | 2.150 | 1419871 | 2371 | 0.866 |
| 5 | 2.150 | 1420342 | 2377 | 0.902 |
| 6 | 2.142 | 1422675 | 2376 | 0.897 |
| Mean | 2.151 | 1420436 | 2379.833 | 0.877 |
| SD | 0.006 | 5615.921 | | |
| RSD(%) | 0.279 | 0.395 | | |

**Table 6.24B. System suitability of Method 1 for MOX**

| Sr No | Parameters | Acceptance Criteria | MOX |
|---|---|---|---|
| 1 | Theoretical Plates | >1000 | 2379.8 |
| 2 | Tailing factor | <2 | 0.877 |
| 3 | RSD of area | <2% | 0.395 |
| 4 | RSD of Ret.Time | <1% | 0.279 |

**Linearity:** The response was linear from 10-60 µg/mL with $R^2$ value of 0.999. Linearity graph is presented as Fig.6.6.

**Table 6.25 Linearity data for MOX: Peak areas for Concentration range**

| Conc(µg/mL) | Peak Area* |
|---|---|
| 10 | 741934.7 |
| 20 | 1419910 |
| 30 | 2111037 |
| 40 | 2754234 |
| 50 | 3459527 |
| 60 | 4034960 |

*Average of three injections

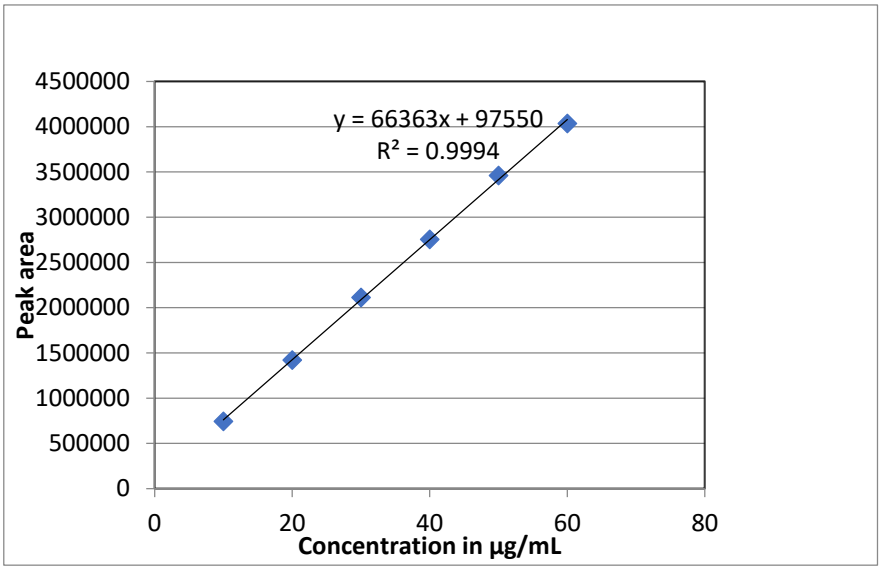



**Fig. 6.6. Linearity Graph for MOX (Method 1)**

Limit of Detection and Limit of Quantitation were calculated and found to be:

Limit of Detection, LOD (calculated) =1.57 µg/mL

Limit of Quantitation, LOQ (calculated) =5.22 µg/mL

**Precision Study of Analysis of MOX by Method 1:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of the tablet extract on two different days.

Acceptance criteria: % RSD should be <2%.

Conc calculated from the linearity graphs and equations obtained on the respective days.

Fresh solutions were prepared each day and intraday precision calculated.

**Table 6.26. Inter- and intraday Precision for MOX (Conc. 20 µg/mL)**

| Day 1 | | | Day 2 | | |
|---|---|---|---|---|---|
| Sr. No. | Peak Area | Conc (µg/mL) | Sr. No. | Peak Area | Conc (µg/mL) |
| 1 | 1420471 | 19.935 | 1 | 1430356 | 20.084 |
| 2 | 1428320 | 20.053 | 2 | 1429863 | 20.076 |
| 3 | 1410939 | 19.791 | 3 | 1428876 | 20.061 |
| 4 | 1419871 | 19.926 | 4 | 1422456 | 19.965 |
| 5 | 1420342 | 19.933 | 5 | 1423465 | 19.980 |
| 6 | 1422675 | 19.968 | 6 | 1428976 | 20.063 |
| Mean | 1420436 | 19.934 | Mean | 1427332 | 20.038 |
| SD | 5615.921 | 0.085 | SD | 3445.759 | 0.052 |
| RSD(%) | 0.395 | 0.425 | RSD(%) | 0.241 | 0.259 |
| Inter-day Precision | | | | | |
| Mean | 1423884 | 19.986 | | | |
| SD | 5718.469 | 0.08617 | | | |
| RSD(%) | 0.402 | 0.431 | | | |

**Accuracy:**

Accuracy (% Recovery) was assessed in triplicate, at three different concentration levels equivalent to 80, 100 and 120% of the target concentration of active ingredient, by spiking a known amount of each of the standard to a sample of known concentration and then calculating the recovery and RSD (%) for each concentration as per procedure given in section 5.3.1.4.7 (pp 129).

**Robustness**: The effect of changes in mobile phase proportion, pH and flow rate on retention time, peak area, number of theoretical plates and peak symmetry was studied.

**Effect of mobile phase proportion**: Ratios of mobile phase, namely, 72:28 and 68:32 of methanol: buffer as compared to the optimized ratio of 70:30 used in the method.

**Table 6.27. Results of Accuracy of Method 1 for MOX**

| Level of addition (std) (%) | Amount of std added (µg/mL) | Avg. Peak Area | Conc (µg/mL) | Assay | Recovery | % recovery |
|---|---|---|---|---|---|---|
| 80 | 20 | 3052671 | 44.84 | 25.68 | 19.16 | 95.8 |
| 100 | 25 | 3486146 | 51.06 | 25.68 | 25.38 | 101.52 |
| 120 | 30 | 3724623 | 54.70 | 25.68 | 29.02 | 96.73 |

**Table 6.28. Robustness: Effect of Mobile Phase Ratio**

| Mobile Phase Ratio | 72:28 | 70:30 (optimized) | 68:32 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 1412491 | 1419910 | 1433881 | 0.777 |
| %RSD | 0.509 | 0.613 | 0.195 | |

**Effect of variation in pH**: pH of buffer was adjusted to 2.8 and 3.2 as compared to optimized pH of 3.0 used in the method (0.2 units variation)

**Table 6.29. Robustness: Effect of pH**

| pH | 2.8 | 3.0 (optimized) | 3.2 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 1417116 | 1419910 | 1431022 | 0.707 |
| %RSD | 0.637 | 0.613 | 0.645 | |

**Effect of variation in mobile phase flow rate**: Flow rate of mobile phase was changed to 1.05 mL/min and 1.45 mL/min as compared to optimized flow rate of 1.25 mL/min used in the method (0.2 mL variation).

**Table 6.30. Robustness: Effect of Mobile Phase Flow Rate**

| Mobile Phase Flow Rate (mL/min) | 1.05 | 1.25 (optimized) | 1.45 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 1430361 | 1419910 | 1426069 | 0.453 |
| %RSD | 0.192 | 0.613 | 0.021 | |

**Assay of Marketed Formulation:**

The proposed HPLC method was extended for the determination of MOX in tablets. The tablet used was Mahoflox 400 (batch no. C5ABP010) manufactured by Mankind Pharma Ltd. It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 1. Sample concentration of 25 µg/mL was injected into the chromatograph and resulting peak area from chromatogram recorded.

Average area (6 injections) for assay solution was calculated to be 1801520.
The concentration of sample MOX sample was calculated to be 25.68 µg/mL and assay results found to be complying 103.17 % with label claim (I.P. limits 90-110%).

### 6.2.1.1.4 Norfloxacin

**Table 6.31A. System suitability of Method 1 for NOR (Conc 20 µg/mL)**

| Sr. No. | Rt (min) | Peak Area | N | Tailing Factor |
|---|---|---|---|---|
| 1 | 2.108 | 726638 | 2826 | 0.976 |
| 2 | 2.108 | 736164 | 2838 | 0.980 |
| 3 | 2.108 | 739297 | 2876 | 0.942 |
| 4 | 2.117 | 740136 | 2888 | 0.925 |
| 5 | 2.108 | 740012 | 2828 | 0.968 |
| 6 | 2.108 | 739987 | 2830 | 0.971 |
| Mean | 2.110 | 737039 | 2847.667 | 0.960 |
| SD | 0.004 | 5313.489 | | |
| RSD(%) | 0.017 | 0.721 | | |

**Table 6.31B. System suitability of Method 1 for NOR**

| Sr No | Parameters | Acceptance Criteria | NOR |
|---|---|---|---|
| 1 | Theoretical Plates | >1000 | 2847.667 |
| 2 | Tailing factor | <2 | 0.960 |
| 3 | RSD of area | <2% | 0.721 |
| 4 | RSD of Ret.Time | <1% | 0.017 |

**Linearity:** The response was linear from 10-60 µg/mL with a $R^2$ value of 0.999. Linearity graph is presented as Fig.6.7.

**Table 6.32. Linearity data for NOR: Peak areas for Concentration range**

| Conc(µg/mL) | Peak Area* |
|---|---|
| 10 | 365431.3 |
| 20 | 734033 |
| 30 | 1117134 |
| 40 | 1488039 |
| 50 | 1850903 |
| 60 | 2204221 |

*Average of three injections

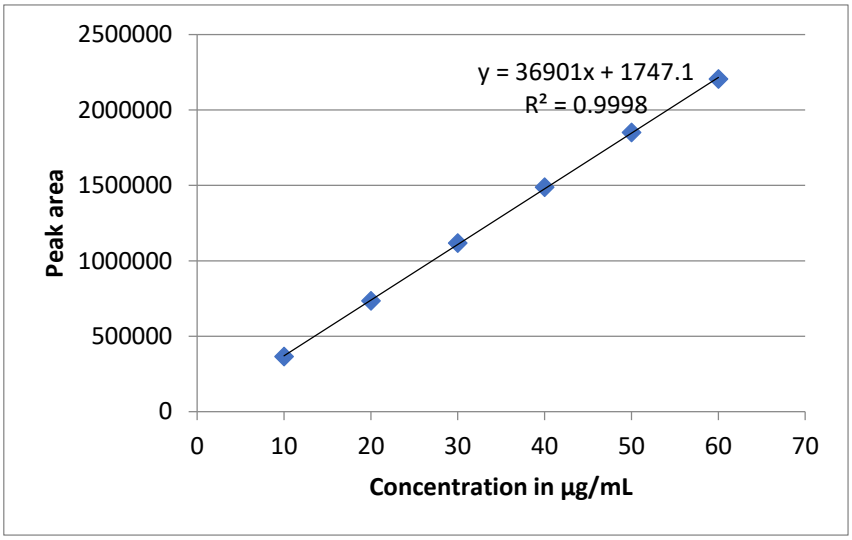



**Fig. 6.7. Linearity Graph for NOR (Method 1)**

Limit of Detection and Limit of Quantitation were calculated and found to be:

Limit of Detection, LOD (calculated) =0.799 µg/mL

Limit of Quantitation, LOQ (calculated) =2.677 µg/mL

**Precision Study of Analysis of NOR by Method 1:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of the tablet extract on two different days.

Acceptance criteria: % RSD should be <2%.

**Accuracy:**

Accuracy (% Recovery) was assessed in triplicate, at three different concentration levels equivalent to 80, 100 and 120% of the target concentration of active ingredient, by spiking a known amount of each of the standard to a sample of known concentration (30 µg/mL) and then calculating the recovery and RSD (%) for each concentration as per procedure given in section 5.3.1.4.7 (pp 129).

**Table 6.33. Inter- and intraday Precision for NOR (Conc. 20 µg/mL)**

| Day 1 | | | Day 2 | | |
|---|---|---|---|---|---|
| Sr. No. | Peak Area | Conc(µg/mL) | Sr. No. | Peak Area | Conc(µg/mL) |
| 1 | 726638 | 19.644 | 1 | 741034 | 20.034 |
| 2 | 736164 | 19.902 | 2 | 740124 | 20.010 |
| 3 | 739297 | 19.987 | 3 | 739968 | 20.005 |
| 4 | 740136 | 20.010 | 4 | 741123 | 20.037 |
| 5 | 740012 | 20.007 | 5 | 740344 | 20.016 |
| 6 | 739987 | 20.006 | 6 | 743989 | 20.114 |
| Mean | 737039 | 19.926 | Mean | 741097 | 20.036 |
| SD | 5313.489 | 0.144 | SD | 1493.766 | 0.041 |
| RSD(%) | 0.721 | 0.723 | RSD (%) | 0.202 | 0.202 |
| Inter-day Precision | | | | | |
| Mean | 739068 | 19.981 | | | |
| SD | 4282.359 | 0.116 | | | |
| RSD(%) | 0.579 | 0.581 | | | |

**Table 6.34. Results of Accuracy of Method 1 for NOR**

| Level of addition (std) (%) | Amount of std added (µg/ml) | Avg. Peak Area | Conc (µg/ml) | Assay | Recovery | % recovery |
|---|---|---|---|---|---|---|
| 80 | 24 | 2037652 | 55.15 | 30.632 | 24.518 | 102.16 |
| 100 | 30 | 2287631 | 61.85 | 30.632 | 31.218 | 104.06 |
| 120 | 36 | 2507652 | 67.41 | 30.632 | 36.778 | 102.16 |

**Robustness**: The effect of changes in mobile phase proportion, pH and flow rate on retention time, peak area, number of theoretical plates and peak symmetry was studied.

**Effect of mobile phase proportion**: Mobile phase ratios of 72:28 and 68:32 of methanol: buffer as compared to optimized ratio of 70:30 used in the method were tried.

**Table 6.35. Robustness: Effect of Mobile Phase Ratio**

| Mobile Phase Ratio | 72:28 | 70:30 (optimized) | 68:32 | % RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 736999 | 734033 | 723961.7 | 1.067 |
| % RSD | 0.824 | 0.898 | 0.669 | |

**Effect of variation in pH**: pH of buffer was adjusted to 2.8 and 3.2 as compared to optimized pH of 3.0 used in the method (0.2 units variation)

**Table 6.36. Robustness: Effect of pH**

| pH | 2.8 | 3.0 (optimized) | 3.2 | % RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 731268 | 734033 | 722494.3 | 0.950 |
| %RSD | 0.581 | 0.898 | 0.640 | |

**Effect of variation in mobile phase flow rate**: Flow rate of mobile phase was changed to 1.05 mL/min and 1.45 mL/min as compared to optimized flow rate of 1.25 mL/min used in the method (0.2 mL variation).

**Table 6.37. Robustness: Effect of Mobile Phase Flow Rate**

| Mobile Phase Flow Rate (mL/min) | 1.05 | 1.25 (optimized) | 1.45 | % RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 731570.7 | 734033 | 732180.3 | 0.586 |
| %RSD | 0.5196 | 0.898 | 0.452 | |

**Assay of Marketed Formulation:**

The proposed HPLC method was extended for the determination of NOR in tablets. The tablet used was Norflox- 400 (batch no. ACT6163) manufactured by Cipla Ltd. It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 1. Sample concentration of 20 µg/mL was injected into the chromatograph and resulting peak area from chromatogram recorded.

Average area (6 injections) for assay solution was calculated to be 798022.5.

The concentration of sample NOR sample was calculated to be 21.58 µg/mL and assay results found to be complying 106.75 % with label claim (I.P. limits 90-110%).

**6.2.1.1.5 Ofloxacin**

**Table 6.38A. System suitability of Method 1 for OFL (20 µg/mL)**

| Sr. No. | Rt (min) | Peak Area | N | Tailing Factor |
|---|---|---|---|---|
| 1 | 2.100 | 1538574 | 4016 | 0.877 |
| 2 | 2.092 | 1539239 | 4056 | 0.877 |
| 3 | 2.092 | 1537185 | 4069 | 0.881 |
| 4 | 2.092 | 1543172 | 4074 | 0.874 |
| 5 | 2.092 | 1531558 | 4081 | 0.880 |
| 6 | 2.092 | 1539273 | 4057 | 0.873 |
| Mean | 2.093 | 1538167 | 4058.833 | 0.877 |
| SD | 0.003 | 3800.824 | | |
| RSD(%) | 0.156 | 0.247 | | |

**Table 6.38B. System suitability of Method 1 for OFL**

| | Parameters | Acceptance Criteria | OFL |
|---|---|---|---|
| 1 | Theoretical Plates | >1000 | 4058.8 |
| 2 | Tailing factor | <2 | 0.877 |
| 3 | RSD of area | <2% | 0.247 |
| 4 | RSD of Ret. Time | <1% | 0.156 |

**Linearity:** The response was linear for a range of concentrations of OFL from 10-60 µg/mL with $R^2$ value of 0.999. Linearity graph is shown as Fig. 6.8.

**Table 6.39. Linearity data for OFL: Peak areas for Concentration range**

| Conc(µg/mL) | Peak Area* |
|---|---|
| 10 | 706297.7 |
| 20 | 1337761 |
| 30 | 1992044 |
| 40 | 2619663 |
| 50 | 3228983 |
| 60 | 3799010 |

*Average of three injections

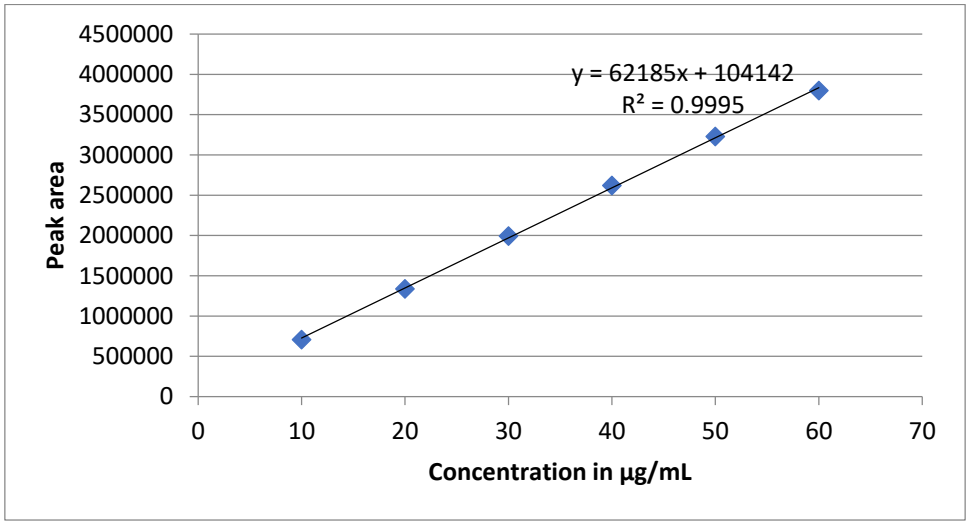



**Fig. 6.8. Linearity Graph for OFL (Method 1)**

Limit of Detection and Limit of Quantitation were calculated and found to be:

Limit of Detection, LOD (calculated) =1.39 µg/mL

Limit of Quantitation, LOQ (calculated) =4.64 µg/mL

**Precision Study of Analysis of OFL by Method 1:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of dilutions made from the tablet extract on two different days.

Acceptance criteria: % RSD should be <2%.

**Table 6.40. Inter- and intraday Precision for OFL (Conc. 25 µg/mL)**

| Day 1 | | | Day 2 | | |
|---|---|---|---|---|---|
| Sr. No. | Peak Area | Conc (µg/mL) | Sr. No. | Peak Area | Conc (µg/mL) |
| 1 | 1538574 | 24.574 | 1 | 1545942 | 25.555 |
| 2 | 1539239 | 24.585 | 2 | 1538970 | 25.439 |
| 3 | 1537185 | 24.552 | 3 | 1530987 | 25.306 |
| 4 | 1543172 | 24.648 | 4 | 1544269 | 25.528 |
| 5 | 1531558 | 24.462 | 5 | 1539873 | 25.454 |
| 6 | 1539273 | 24.586 | 6 | 1567364 | 25.913 |
| Mean | 1538167 | 24.568 | Mean | 1544568 | 25.533 |
| SD | 3800.824 | 0.061 | SD | 12324.44 | 0.206 |
| RSD (%) | 0.247 | 0.249 | RSD (%) | 0.798 | 0.807 |
| Inter-day Precision | | | | | |
| Mean | 1541367 | 25.050 | | | |
| SD | 9315.656 | 0.524 | | | |
| RSD (%) | 0.60476 | 2.092 | | | |

**Accuracy:**

Accuracy (% Recovery) was assessed in triplicate, at three different concentration levels equivalent to 80, 100 and 120% of the target concentration of active ingredient, by spiking a known amount of each of the standard to a sample of known concentration and then

calculating the recovery and RSD (%) for each concentration as per procedure given in section 5.3.1.4.7(pp 129).

**Table 6.41. Results of Accuracy of Method 1 for OFL**

| Level of addition (std) (%) | Amount of std added (µg/ml) | Avg. Peak Area | Conc. (µg/ml) | Assay | Recovery | % recovery |
|---|---|---|---|---|---|---|
| 80 | 20 | 2748604 | 44.03 | 24.57 | 19.46 | 97.30 |
| 100 | 25 | 3171021 | 50.82 | 24.57 | 26.25 | 105.00 |
| 120 | 30 | 3337886 | 53.51 | 24.57 | 28.94 | 96.47 |

**Robustness**: The effect of changes in mobile phase proportion, pH and flow rate on retention time, peak area, number of theoretical plates and Tailing Factor was studied.

**Effect of mobile phase proportion**: Ratios 72:28 and 68:32 of methanol: buffer as compared to optimized ratio of 70:30 used in the method were tried.

**Table 6.42. Robustness: Effect of Mobile Phase Ratio**

| Mobile Phase Ratio | 72:28 | 70:30 (optimized) | 68:32 | %RSD |
|---|---|---|---|---|
| Avg.Peak Area (n=3) | 1334310 | 1337761 | 1331447 | 0.284 |
| %RSD | 0.225 | 0.115 | 0.300 | |

**Effect of variation in pH**: pH of buffer was adjusted to 2.8 and 3.2 as compared to optimized pH of 3.0 used in the method (0.2 units variation)

**Table 6.43. Robustness: Effect of pH**

| pH | 2.8 | 3.0 (optimized) | 3.2 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 1342680 | 1337761 | 1333941 | 0.348 |
| %RSD | 0.361 | 0.115 | 0.138 | |

**Effect of variation in mobile phase flow rate:** Flow rate of mobile phase was changed to 1.05 mL/min and 1.45 mL/min as compared to optimized flow rate of 1.25 mL/min used in the method (0.2 mL variation).

**Table 6.44. Robustness: Effect of Mobile Phase Flow Rate**

| Mobile Phase Flow Rate (mL/min) | 1.05 | 1.25 (optimized) | 1.45 | %RSD |
|---|---|---|---|---|
| Avg. Peak Area (n=3) | 1339177 | 1337761 | 1338075 | 0.263 |
| %RSD | 0.476 | 0.115 | 0.168 | |

**Assay of Marketed Formulation:**

The proposed HPLC method was extended for the determination of OFL in tablets. The tablet used was Zenflox- 400 (batch no. E1AH0004) manufactured by Mankind Pharma Ltd. It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 1. Sample concentration of 25 µg/mL was injected into the chromatograph and resulting peak area from chromatogram recorded.

Average area (6 injections) for assay solution was calculated to be 1538167.

The concentration of sample OFL sample was calculated to be 24.568 µg/mL and assay results found to be complying 98.004 % with label claim (I.P. limits 90-110%).

The system suitability and validation results are summarized in Table 6.45.

**Table 6.45. System Suitability and Validation parameters using proposed Method 1 using mobile phase Methanol: phosphate buffer pH 3.0, in the ratio 70:30, at flow rate 1.25 mL/min, and column temperature 40 °C**

| Parameter | CIP | LEV | MOX | NOR | OFL |
|---|---|---|---|---|---|
| N | 2327 | 3793 | 2380 | 2848 | 4059 |
| Tailing factor (Pk sym) | 0.891 | 0.889 | 0.877 | 0.960 | 0.877 |
| RSD of area (< 2%) | 0.998 | 0.123 | 0.395 | 0.721 | 0.247 |
| $R_t$(min) | 2.095 | 2.095 | 2.151 | 2.110 | 2.093 |
| RSD of $R_t$ (<2%) | 0.197 | 0.197 | 0.279 | 0.017 | 0.156 |
| Linearity | y = 852.15x + 2198<br>$R^2$ = 0.9992 | y = 69819x + 134352<br>$R^2$ = 0.9994 | y = 66363x + 97550<br>$R^2$ = 0.9994 | y = 36901x + 1747.1<br>$R^2$ = 0.9998 | y = 62185x + 104142<br>$R^2$ =0.9995 |
| LOD(µg/mL) | 1.79 | 1.57 | 1.57 | 0.80 | 1.39 |
| LOQ(µg/mL) | 5.96 | 5.24 | 5.22 | 2.68 | 4.64 |
| Precision (RSD < 2%) | Complies | Complies | Complies | Complies | Complies |
| Assay of marketed formulation (Limits 90-110%) | 100.05% | 104.19% | 103.17% | 106.75% | 98.004% |
| Accuracy | 101-102% | 95-102% | 95-102% | 102-105% | 96-105% |
| Robustness (RSD < 2%) | Complies | Complies | Complies | Complies | Complies |

**Evaluation of Stability Indicating Capability of Method 1**:

As seen in previous sections, Method 1 has been successfully applied to all selected FQs. However, when method was applied for stress induced study, a degradant of LEV formed under experimental conditions of acid hydrolysis (as mentioned in 5.3.1.5, pp 132) eluted very close to the drug peak and hence resolution was not satisfactory. The degradant has been termed as LDA and the chromatogram of LEV degraded through acid hydrolysis is presented as Fig. 6.9.

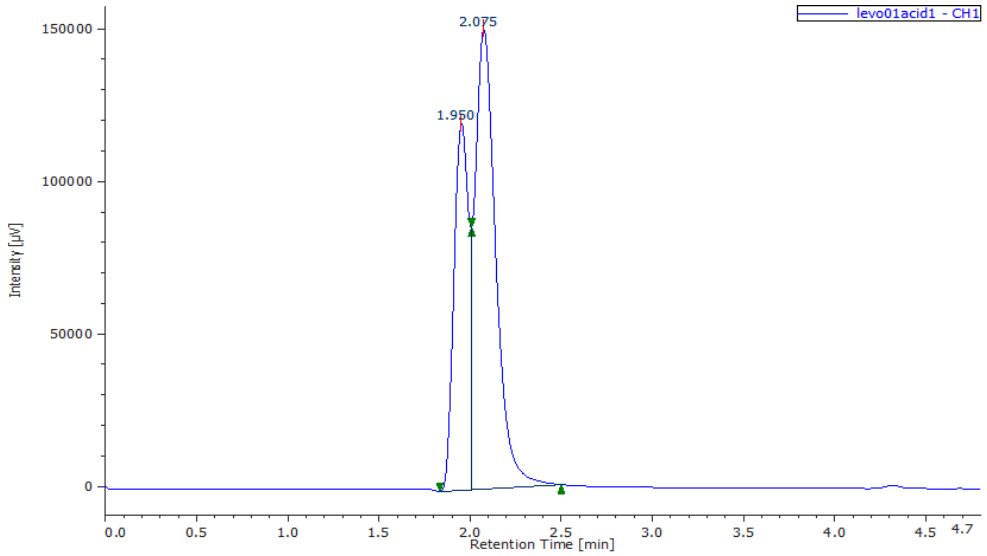



**Fig. 6.9. Chromatogram of LEV subjected to stress conditions of acid hydrolysis analyzed by Method 1**

Hence attempts were made to develop alternate method that can resolve the drug peak from its degradants (LDO and LDA).

### 6.2.2. Method 2

#### 6.2.2.1. Trials

Series of trials were conducted for resolution of drug peak from its degradants (LDO and LDA). Results of the study are summarised in Table 6.46. Some chromatograms have been presented in Fig 6.10, 6.11 and 6.12.

**Table 6.46. Trials for Development of Method for LEV and its Degradants**

| Sr. No. | Trial | Range or Variation | Observations |
|---|---|---|---|
| 1 | Methanol and Phosphate buffer:<br>(a) % of Methanol<br>(b) pH of buffer | <br>95 to 50%<br>2.5 to 3.5 | Peak of degradant not resolved<br>Poor Tailing Factor and N |
| 2 | Methanol and Phosphate buffer with 0.4% TEA, pH adjusted with OPA.<br>(a) % of Methanol<br>(b) pH of buffer | <br>95 to 50%<br>2.5 to 3.5 | Peak of degradant not resolved |
| 3 | Methanol and 0.1% OPA<br>(a) % of Methanol | <br>95 to 50% | Peak of degradant not resolved (Representative chromatogram in Fig.6.10) |
| 4 | Higher conc of drug | 20 and 40 µg/mL | N increased tremendously |
| 5 | Change in flow rate | 0.8 to 1.25 mL/min | No improvement in tailing |
| 6 | Methanol and Sodium buffer (20mM $Na_2HPO_4$) | Methanol: Phosphate buffer pH 3.0, 0.8 mL/min | Good separation at 40:60 but high tailing |
| 7 | Trials with 0.1% TEA | Methanol: Phosphate buffer pH 3.0 (43:57), 0.8 mL/min | Least tailing and high N and good resolution |
| 8 | Potassium and sodium phosphate buffers | Methanol with 20mM of each buffer (43:57) | Sodium buffer (20mM $Na_2HPO_4$) shows higher N and lower tailing |

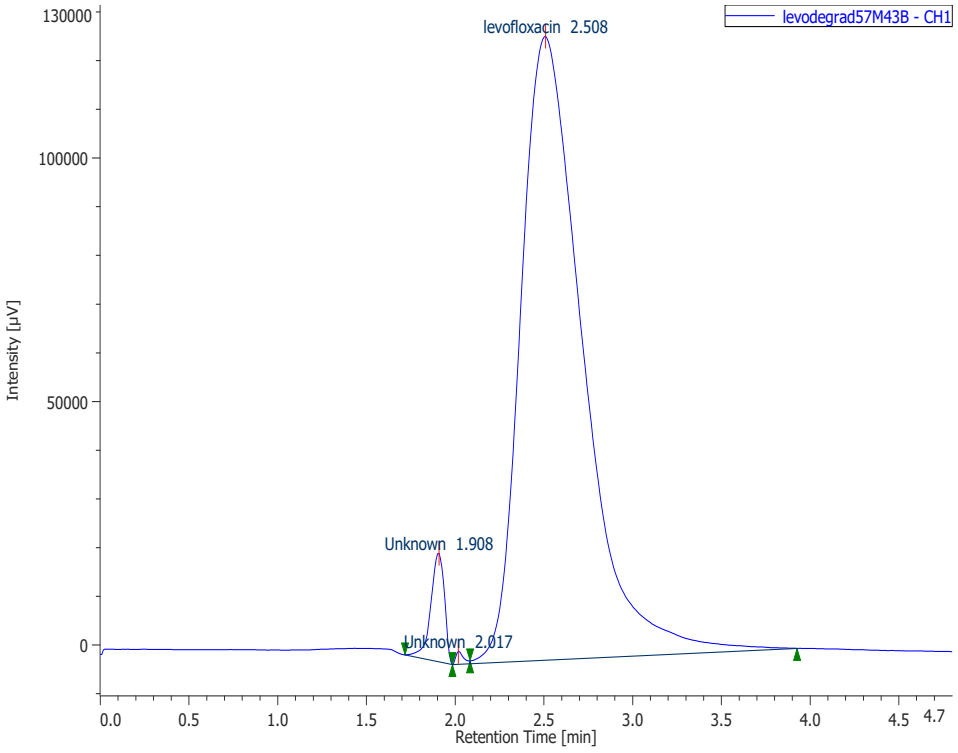



**Fig.6.10. Chromatogram of acid hydrolysis degraded product of LEV (LDA) at 57:43 (methanol: 0.1%OPA); 1.0 mL/min**

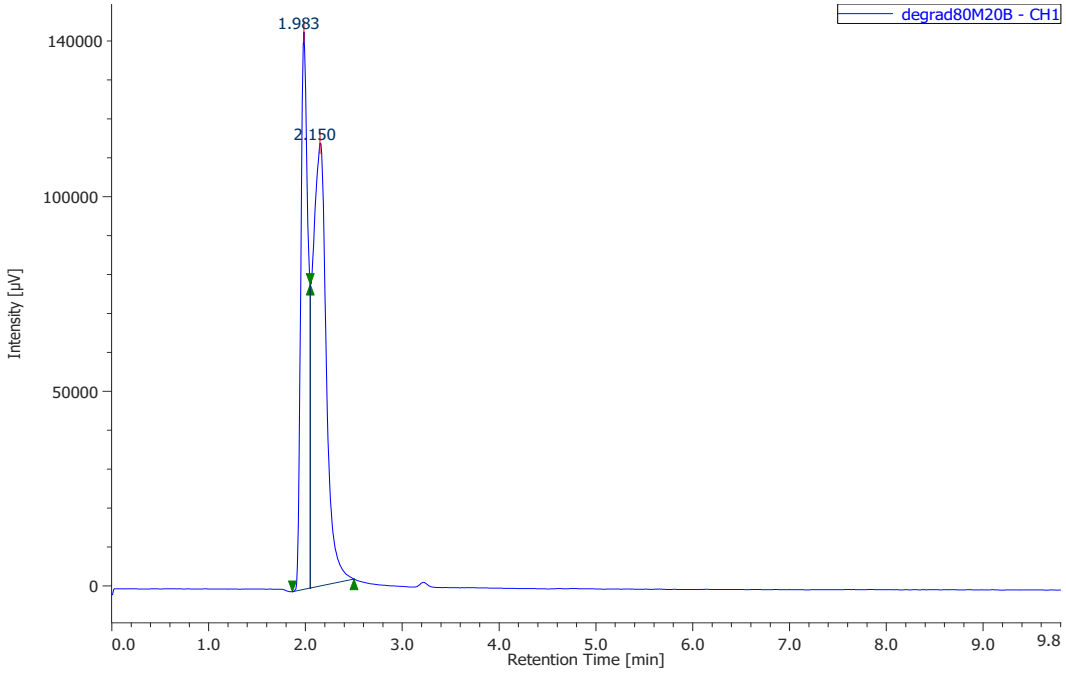



**Fig.6.11. Chromatogram of acid hydrolysis degraded product of LEV (LDA) at 80:20 (methanol: phosphate buffer pH 3.0)**

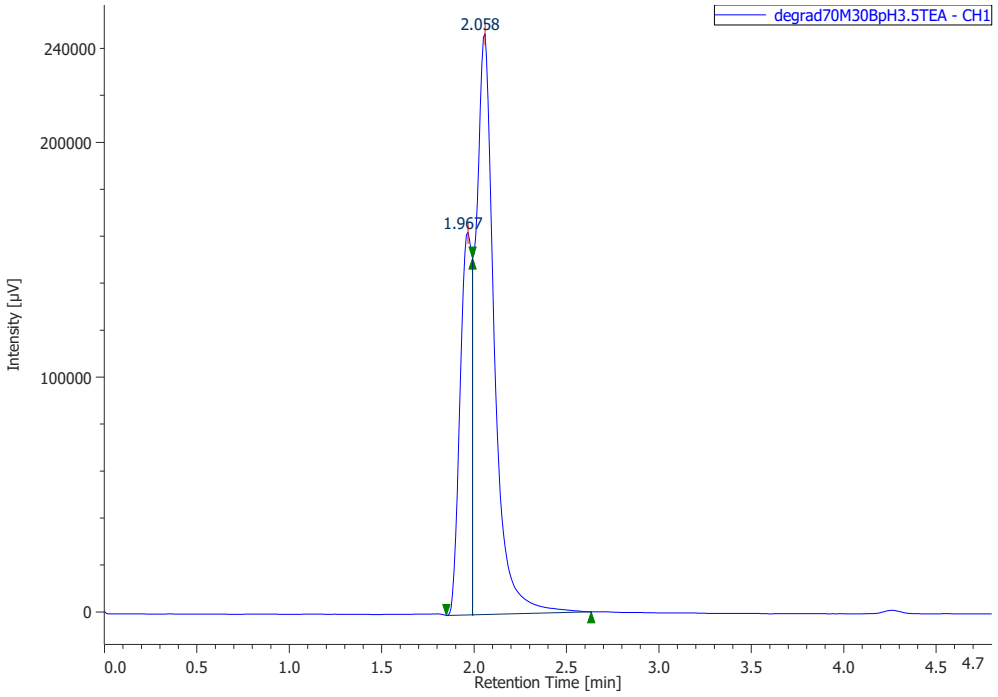



**Fig.6.12. Chromatogram of degraded product acid hydrolysis degraded product of LEV (LDA) at 70:30 (methanol: phosphate buffer with 0.1% TEA, pH 3.5)**

Mobile phase of Methanol: phosphate buffer 20 mM, adjusted to pH 3.0, at a flow rate of 1.25 mL/min, and detector wavelength 294 nm showed promising results but the N was found to be low and hence attempts were made to improve N. The options for increasing N [365] were:

(1) Increasing concentration of the drug

(2) Decreasing flow rate

(3) Increasing proportion of aqueous phase

(4) Trying different column: changing column packing, particle size, length of column

**6.2.2.1.1 Trial 1:** Comparison between Sodium and Potassium phosphate Buffers

Suitability of buffer was tried by comparing between Sodium and Potassium buffers. Methanol: 20 mM phosphate buffer (Sodium or Potassium), adjusted to pH 3.0, at flow rate of 0.8 mL/min, and detector wavelength set at 294 nm.

The results are presented in Table 6.47.

**Table 6. 47. Comparison between Na and K Buffers using Mobile Phase Methanol: 20mM phosphate buffer (Sodium or Potassium), adjusted to pH 3.0, at 0.8 mL/min**

| Buffer | Sodium phosphate buffer | | | | Potassium phosphate buffer | | | |
|---|---|---|---|---|---|---|---|---|
| | LDA | | LDO | | LDA | | LDO | |
| | LDA | LEV | LEV | LDO | LDA | LEV | LEV | LDO |
| Rs | 2.097 | ----- | 2.669 | 3.759 | 2.088 | ------ | 2.985 | 3.910 |
| N | 3188 | 3220 | 2752 | 3056 | 3068 | 2828 | 2711 | 2895 |
| PS | 1.383 | 1.600 | 1.656 | 1.567 | 1.448 | 1.571 | 1.688 | 1.419 |

It was observed that Sodium phosphate ($Na_2HPO_4$) buffer showed higher N and lower tailing in most cases.

**6.2.2.1.2 Trial 2: Effect of Flow Rate**

Effect of flow rate was checked by comparing between flow rates of 0.8, 1.0 and 1.25 mL/min. Methanol: 20 mM Sodium phosphate buffer, adjusted to pH 3.0, at different flow rates of 0.8 to 1.25 mL/min, and detector wavelength set at 294 nm.

The results are presented in Table 6.48.

**Table 6.48. Effect of Flow Rate using Mobile Phase Methanol: 20 mM Sodium phosphate buffer, adjusted to pH 3.0 on LDA**

| Flow rate | 0.8 mL/min | | 1.0 mL/min | | 1.25 mL/min | |
|---|---|---|---|---|---|---|
| | LDA | LEV | LDA | LEV | LDA | LEV |
| Rs | 2.097 | ------ | 2.287 | ------- | 2.351 | ------ |
| N | 3188 | 3220 | 3579 | 3429 | 3073 | 3210 |
| PS | 1.383 | 1.600 | 1.315 | 1.520 | 1.395 | 1.583 |

Though it was observed that Tailing Factor was lowest and N highest at 1.0 mL/min, the $R_t$ was below 3.325 and Vr was 2.66 which is close to $V_0$ (2.5). Hence 0.8 mL/min was selected where Rt and Vr are both 4.133 which is sufficiently higher than $V_0$ of 3.125.

#### 6.2.2.1.3 Trial 3: Effect of Proportion of Mobile Phase

Effect of proportion of mobile phase components was checked by comparing between various ratios of organic and aqueous phases as shown in Tables 6.49 to 6.52.

**Table 6.49. Effect of Proportion of Mobile Phase with Sodium phosphate (buffer at 1.25 mL/min (for LDA, the LEV sample degraded by acid hydrolysis)**

| Mobile Phase Proportion | 45:55 | | 43:57 | | 40:60 | | 38:62 | | 35:65 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LDA | LEV | LDA | LEV | LDA | LEV | LDA | LEV | LDA | LEV |
| Rs | 2.001 | ------ | 2.369 | ------ | 2.898 | ------ | 3.364 | ------ | 4.172 | ------ |
| N | 2841 | 3266 | 2905 | 3323 | 2873 | 3298 | 2808 | 3295 | 2854 | 3387 |
| PS | ------ | 1.611 | ----- | 1.572 | 1.398 | 1.622 | 1.524 | 1.622 | 1.465 | 1.649 |

**Table 6.50. Effect of Proportion of Mobile Phase with Potassium phosphate buffer at 1.00 mL/min (for LEV sample degraded by acid hydrolysis)**

| Mobile Phase Proportion | 43:57 | | 40:60 | |
|---|---|---|---|---|
| | LDA | LEV | LDA | LEV |
| Rs | 2.036 | ------ | 2.627 | ------ |
| N | 2614 | 2592 | 2658 | 2719 |
| PS | ----- | 1.606 | 1.492 | 1.566 |

**Table 6.51. Effect of Proportion of Mobile Phase with Potassium phosphate buffer at 0.80 mL/min (for LEV sample degraded by acid hydrolysis)**

| Mobile Phase Proportion | 45:55 | | 43:57 | | 40:60 | | 38:62 | | 35:65 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LDA | LEV | LDA | LEV | LDA | LEV | LDA | LEV | LDA | LEV |
| Rs | 1.862 | ------ | 2.088 | ------ | 2.682 | ------ | 3.154 | ------ | 3.849 | ------ |
| N | 3196 | 2981 | 3068 | 2828 | 3083 | 2763 | 2913 | 2936 | 2915 | 2843 |
| PS | ------ | 1.578 | 1.448 | 1.571 | 1.472 | 1.636 | 1.483 | 1.617 | 1.441 | 1.741 |

**Table 6.52. Effect of Proportion of Mobile Phase with Potassium phosphate buffer at 0.80 mL/min (for LEV sample degraded by oxidation)**

| Mobile Phase Proportion | 45:55 | | 43:57 | | 40:60 | | 38:62 | | 35:65 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LEV | LDO | LEV | LDO | LEV | LDO | LEV | LDO | LEV | LDO |
| Rs | 2.651 | 3.999 | 2.985 | 3.910 | 3.754 | 3.399 | 4.455 | 2.738 | 5.720 | 1.389 |
| N | 2748 | 2943 | 2711 | 2895 | 2656 | 2984 | 2701 | 3018 | 2711 | 3443 |
| PS | 1.663 | 1.567 | 1.688 | 1.419 | 1.671 | 1.667 | 1.670 | 1.682 | 1.735 | ------ |

Based on above observations, Sodium phosphate buffer (20 mM) with 0.1% TEA adjusted to pH 3.0 with OPA was chosen as buffer with preferred flow rate of 0.8 mL/min. The preferred ratio for methanol and buffer was 43:57.

The optimized method resulting from the trials is presented in Table 6.53 and resultant chromatograms are presented in Fig. 6.13 & 6.14.

**Table 6.53. Experimental Variables for Proposed Method 2.**

| Method No. | Column | Column Temperature | Mobile Phase | Flow rate (mL/min) | Applicable to |
|---|---|---|---|---|---|
| 2 | C18 | 40 °C | Methanol: Phosphate buffer with 0.1% TEA, adjusted to pH 3.0 with OPA (43:57) | 0.8 | Acid and oxidative degradants of LEV |

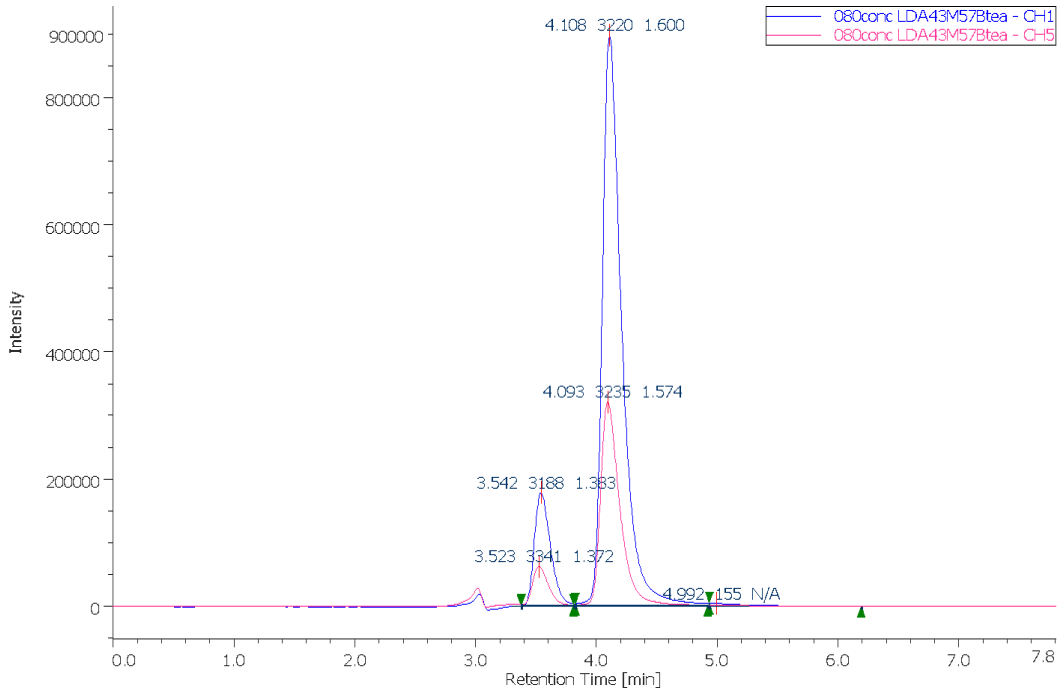



**Fig. 6.13. Chromatogram of LEV degraded by acid hydrolysis using Method 2**

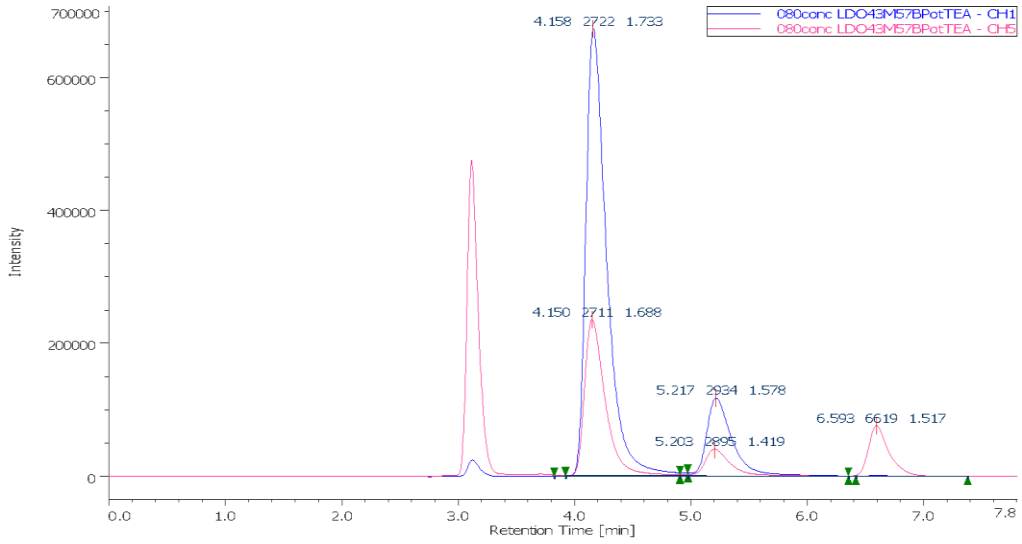



**Fig. 6.14. Chromatogram of LEV degraded by oxidation using Method 2**

### 6.2.2.2. System Suitability and Validation of Method 2

The system suitability and validation parameters achieved through Method 2, following procedures as per section 5.3.2.1(pp 137) have been reported here.

LDA is the degradant produced through acid hydrolysis of LEV and LDO the degradant in sample of LEV exposed to oxidative degradation.

**Table 6.54. System Suitability Parameters using proposed Method 2 {Methanol: Phosphate buffer with 0.1% TEA, adjusted to pH 3.0 with orthophosphoric acid (OPA) in the ratio 43:57, at a flow rate of 0.8 mL/min with column at 40 °C}**

| System Suitability | LEV | LDO | LDA |
|---|---|---|---|
| N | 3419 | 3497 | 3291 |
| Tailing factor (Pk sym) | 1.502 | 1.419 | 1.368 |
| % RSD of area (<2%) | 0.577 | 1.068 | 0.546 |
| $R_t$ | 4.118 | 5.041 | 3.545 |
| % RSD of $R_t$(<2%) | 0.434 | 0.280 | 0.284 |
| $R_s$ with adjacent peak | 2.932 | 4.565 | 2.169 |

**Precision Study for Analysis of LEV in presence of degradant:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of the tablet extract on two different days.

Acceptance criteria: % RSD should be <2%.

**Tables 6.55(1), (2), (3) & (4). Precision Study Data of LEV sample degraded by acid hydrolysis**

**(1) Precision Study Data for Peak 1 (LDA), on Day 1**

| Sr. No. | Rt | A | H | N | $R_s$ | Peak Sym |
|---|---|---|---|---|---|---|
| 1 | 3.525 | 1850853 | 202297 | 3336 | 2.119 | 1.392 |
| 2 | 3.542 | 1855699 | 197219 | 3213 | 2.164 | 1.392 |
| 3 | 3.550 | 1864015 | 197916 | 3230 | 2.205 | 1.401 |
| 4 | 3.550 | 1874776 | 200106 | 3295 | 2.183 | 1.336 |
| 5 | 3.550 | 1854599 | 197444 | 3282 | 2.158 | 1.374 |
| 6 | 3.550 | 1873447 | 200535 | 3392 | 2.186 | 1.315 |
| Mean | 3.545 | 1862232 | 199252.8 | 3291.333 | 2.169 | 1.368 |
| SD | 0.010 | 10165.94 | 2041.288 | 66.524 | 0.030 | 0.035 |
| %RSD | 0.284 | 0.546 | 1.024 | 2.021 | 1.371 | 2.555 |

**(2) Precision Study Data for Peak 2 (LEV), on Day 1**

| Sr. No. | Rt | A | H | N | PS |
|---|---|---|---|---|---|
| 1 | 4.083 | 11396230 | 1010029 | 3332 | 1.508 |
| 2 | 4.117 | 11273799 | 1002865 | 3420 | 1.541 |
| 3 | 4.133 | 11295257 | 1002260 | 3506 | 1.458 |
| 4 | 4.125 | 11365399 | 1015362 | 3487 | 1.515 |
| 5 | 4.125 | 11220466 | 1001874 | 3352 | 1.514 |
| 6 | 4.125 | 11349090 | 1015067 | 3418 | 1.475 |
| Mean | 4.118 | 11316706.833 | 1007909.500 | 3419.167 | 1.502 |
| SD | 0.018 | 65279.409 | 6403.951 | 69.646 | 0.030 |
| %RSD | 0.434 | 0.577 | 0.635 | 2.037 | 2.005 |

**(3) Precision Study Data for Peak 1 (LDA), on Day 2**

| Sr. No. | Rt | A | H | N | Rs | PS |
|---|---|---|---|---|---|---|
| 1 | 3.533 | 1916747 | 209061 | 3638 | 2.052 | 1.333 |
| 2 | 3.558 | 1931929 | 204305 | 3338 | 2.104 | 1.331 |
| 3 | 3.558 | 1929002 | 205149 | 3308 | 2.147 | 1.352 |
| 4 | 3.558 | 1924653 | 205722 | 3505 | 2.177 | 1.332 |
| 5 | 3.558 | 1922497 | 205195 | 3493 | 2.143 | 1.326 |
| 6 | 3.550 | 1920635 | 204987 | 3361 | 2.156 | 1.387 |
| Mean | 3.553 | 1924243.833 | 205736.500 | 3440.500 | 2.130 | 1.344 |
| SD | 0.010 | 5552.422 | 1691.262 | 126.732 | 0.045 | 0.023 |
| %RSD | 0.284 | 0.289 | 0.822 | 3.684 | 2.111 | 1.720 |

**(4) Study Data for Peak 2 (LEV), on Day 2**

| Sr. No. | Rt | A | H | N | PS |
|---|---|---|---|---|---|
| 1 | 4.067 | 11485925 | 1024837 | 3247 | 1.485 |
| 2 | 4.117 | 11457609 | 1023988 | 3353 | 1.491 |
| 3 | 4.125 | 11428585 | 1027241 | 3465 | 1.460 |
| 4 | 4.117 | 11392718 | 1031440 | 3650 | 1.495 |
| 5 | 4.117 | 11388008 | 1028553 | 3454 | 1.476 |
| 6 | 4.117 | 11386676 | 1029454 | 3441 | 1.444 |
| Mean | 4.110 | 11423253.500 | 1027585.500 | 3435.000 | 1.475 |
| SD | 0.021 | 41590.907 | 2824.870 | 133.858 | 0.020 |
| %RSD | 0.518 | 0.364 | 0.275 | 3.897 | 1.336 |

**Tables 6.56(1), (2), (3) & (4). Precision Study Data of LEV sample degraded by oxidation**

**(1) Precision Study Data for Peak 1 (LEV), on Day 1**

| Sr. No. | Rt | A | H | N | Rs | PS |
|---|---|---|---|---|---|---|
| 1 | 4.107 | 3983396 | 346701 | 3141 | 2.947 | 1.6 |
| 2 | 4.113 | 4074564 | 352557 | 3119 | 2.951 | 1.593 |
| 3 | 4.117 | 4097185 | 354605 | 3137 | 2.954 | 1.565 |
| 4 | 4.113 | 4091077 | 353401 | 3095 | 2.932 | 1.578 |
| 5 | 4.107 | 4102123 | 354592 | 3120 | 2.915 | 1.565 |
| 6 | 4.100 | 4098213 | 354781 | 3110 | 2.890 | 1.565 |
| Mean | 4.110 | 4074426.333 | 352772.833 | 3120.333 | 2.932 | 1.578 |
| SD | 0.006 | 45639.135 | 3098.621 | 17.061 | 0.025 | 0.0156 |
| %RSD | 0.147 | 1.120 | 0.878 | 0.547 | 0.852 | 0.988 |

**(2) Precision Study Data for Peak 2 (LDO), on Day 1**

| Sr. No. | Rt | A | H | N | PS |
|---|---|---|---|---|---|
| 1 | 5.047 | 652370 | 47351 | 3425 | 1.410 |
| 2 | 5.050 | 667322 | 48568 | 3527 | 1.424 |
| 3 | 5.053 | 661528 | 48353 | 3534 | 1.421 |
| 4 | 5.047 | 657559 | 48721 | 3504 | 1.399 |
| 5 | 5.030 | 663461 | 48869 | 3515 | 1.427 |
| 6 | 5.017 | 672413 | 49098 | 3477 | 1.431 |
| Mean | 5.041 | 662442.167 | 48493.333 | 3497.000 | 1.419 |
| SD | 0.014 | 7076.692 | 614.617 | 40.561 | 0.012 |
| %RSD | 0.280 | 1.068 | 1.267 | 1.160 | 0.844 |

**(3) Precision Study Data for Peak 1 (LEV), on Day 2**

| Sr. No. | Rt | A | H | N | Rs | PS |
|---|---|---|---|---|---|---|
| 1 | 4.133 | 3947581 | 332240 | 3000 | 3.098 | 1.572 |
| 2 | 4.163 | 3904494 | 329683 | 3080 | 3.118 | 1.599 |
| 3 | 4.167 | 3895349 | 327422 | 3058 | 3.091 | 1.597 |
| 4 | 4.180 | 3893984 | 326168 | 3058 | 3.126 | 1.595 |
| 5 | 4.177 | 3873968 | 325695 | 3044 | 3.108 | 1.575 |
| 6 | 4.183 | 3893347 | 325533 | 3019 | 3.130 | 1.597 |
| Mean | 4.167 | 3901453.833 | 327790.167 | 3043.167 | 3.112 | 1.589 |
| SD | 0.018 | 24703.106 | 2669.657 | 29.137 | 0.016 | 0.012 |
| %RSD | 0.442 | 0.633 | 0.814 | 0.957 | 0.500 | 0.770 |

**(4) Precision Study Data for Peak 2 (LDO), on Day 2**

| Sr. No. | Rt | A | H | N | PS |
|---|---|---|---|---|---|
| 1 | 5.163 | 905455 | 61933 | 3232 | 1.352 |
| 2 | 5.193 | 928385 | 61983 | 3317 | 1.363 |
| 3 | 5.180 | 909472 | 63065 | 3421 | 1.362 |
| 4 | 5.210 | 932971 | 62890 | 3415 | 1.349 |
| 5 | 5.200 | 907549 | 62703 | 3419 | 1.341 |
| 6 | 5.233 | 909781 | 61032 | 3264 | 1.332 |
| Mean | 5.197 | 915602.167 | 62267.667 | 3344.667 | 1.350 |
| SD | 0.024 | 11868.956 | 765.828 | 85.165 | 0.012 |
| %RSD | 0.466 | 1.296 | 1.230 | 2.546 | 0.891 |

**Tables 6.57 (1) & (2) Precision Study Data of LEV (undegraded) on Day1 & 2**

**(1) Precision Study Data for undegraded LEV, on Day 1 (Conc. 60 µg/mL)**

| Sr. No. | Rt | A | H | N | PS |
|---|---|---|---|---|---|
| 1 | 4.108 | 7299976 | 315124 | 786 | 0.777 |
| 2 | 4.108 | 7337588 | 313228 | 774 | 0.782 |
| 3 | 4.117 | 7356205 | 315138 | 791 | 0.784 |
| 4 | 4.150 | 7440059 | 313438 | 768 | 0.805 |
| 5 | 4.150 | 7423893 | 312972 | 787 | 0.807 |
| 6 | 4.150 | 7405274 | 312357 | 779 | 0.815 |
| Mean | 4.131 | 7377165.833 | 313709.500 | 780.833 | 0.795 |
| SD | 0.022 | 54576.697 | 1159.322 | 8.750 | 0.016 |
| %RSD | 0.523 | 0.740 | 0.370 | 1.121 | 1.995 |

**(2) Precision Study Data for undegraded LEV, on Day 2 (Conc. 60 µg/mL)**

| Sr. No. | Rt | A | H | N | PS |
|---|---|---|---|---|---|
| 1 | 4.200 | 7460948 | 316927 | 805 | 0.828 |
| 2 | 4.200 | 7458806 | 316302 | 801 | 0.826 |
| 3 | 4.200 | 7464312 | 317226 | 804 | 0.827 |
| 4 | 4.167 | 7466699 | 321913 | 794 | 0.828 |
| 5 | 4.167 | 7460917 | 320972 | 785 | 0.830 |
| 6 | 4.175 | 7481183 | 320387 | 806 | 0.831 |
| Mean | 4.185 | 7465477.500 | 318954.500 | 799.167 | 0.828 |
| SD | 0.017 | 8189.464 | 2408.705 | 8.183 | 0.002 |
| %RSD | 0.403 | 0.110 | 0.755 | 1.024 | 0.225 |

**Linearity**: Linearity in response was observed over concentration range 30-210 µg/mL as presented in Table 6.57, with $R^2$ value of 0.999. Linearity graph is presented as Fig. 6.15.

**Table 6.58. Linearity: Peak areas and Concentration of LEV**

| Conc µg/mL | Peak Area | | | | SD | %RSD |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 30 | 3429937 | 3439877 | 3513509 | 3461108 | 45652.226 | 1.319 |
| 60 | 7299976 | 7337588 | 7356205 | 7331256 | 28644.243 | 0.394 |
| 90 | 11011128 | 10993027 | 10970774 | 10991643 | 20212.569 | 0.184 |
| 120 | 14540336 | 14550249 | 14592841 | 14561142 | 27896.001 | 0.192 |
| 150 | 18608251 | 18600599 | 18655448 | 18621433 | 29705.563 | 0.160 |
| 180 | 21861587 | 21919056 | 21941312 | 21907318 | 41138.164 | 0.188 |
| 210 | 25184229 | 25228798 | 25230796 | 25214608 | 26327.657 | 0.104 |

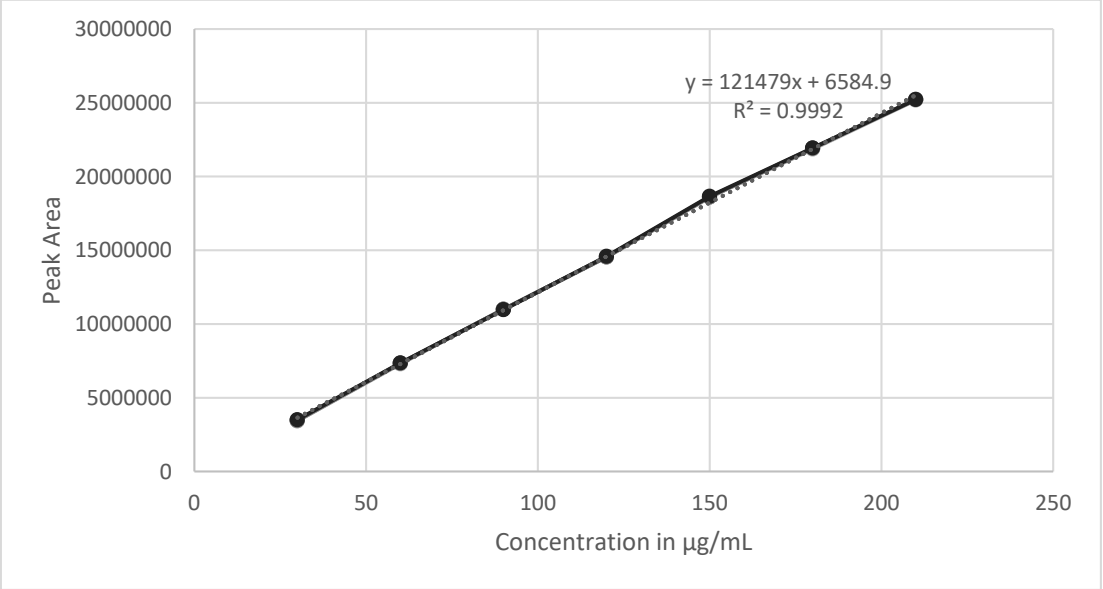



**Fig. 6.15. Linearity Graph for LEV (Method 2)**

Limit of Detection and Limit of Quantitation were calculated and found to be:

Limit of Detection, LOD (calculated) =5.92 µg/mL

Limit of Quantitation, LOQ (calculated) =19.75 µg/mL

**Accuracy:**

Accuracy (% Recovery) was assessed in triplicate, at three different concentration levels of 75, 100 and 125 % of the target concentration of active ingredient, by spiking a known amount of each of the standard to a sample of known concentration and then calculating the recovery and RSD (%) for each concentration as per procedure given under section 5.3.1.4.7 (pp 129).

**Table 6.59. Results of Accuracy for LEV (Method 2)**

| Level of addition (std) (%) | Amount of std added (µg/mL) | Avg. Peak Area | Conc (µg/mL) | Assay | Recovery | % Recovery |
|---|---|---|---|---|---|---|
| 75 | 30 | 10997154 | 90.473 | 60.67 | 29.803 | 99.343 |
| 100 | 60 | 14563509 | 119.831 | 60.67 | 59.161 | 98.601 |
| 125 | 90 | 18625884 | 153.272 | 60.67 | 92.602 | 102.891 |

**Robustness**: The effect of changes in mobile phase proportion, pH and flow rate on retention time, peak area, number of theoretical plates and Tailing Factor was studied.

**Effect of mobile phase proportion:** Mobile phase ratio was changed to 41:59 and 45:55 of methanol: buffer as compared to optimized ratio of 43:57 used in the method.

**Table 6.60. Robustness: Effect of Mobile Phase Ratio**

| Drug/ Degradant | Mobile Phase Ratio | 41:59 | 43:57 (optimized) | 45:55 | % RSD |
|---|---|---|---|---|---|
| LEV | Avg. Peak Area (n=3) | 7739262 | 7565481 | 7555822 | 1.637 |
| | %RSD | 0.352 | 0.468 | 0.422 | |
| LDA | Avg. Peak Area (n=3) | 1863339 | 1925892 | 1909190 | 1.511 |
| | %RSD | 0.378 | 0.418 | 0.306 | |
| LDO | Avg. Peak Area (n=3) | 3039871 | 2962741 | 3056568 | 1.680 |
| | %RSD | 1.225 | 0.329 | 1.175 | |

**Effect of variation in pH:** pH of buffer was adjusted to 2.8 and 3.2 as compared to optimized pH of 3.0 used in the method (0.2 units variation)

**Table 6.61. Robustness: Effect of pH**

| Drug/ Degradant | pH | 2.8 | 3.0 (optimized) | 3.2 | % RSD |
|---|---|---|---|---|---|
| LEV | Avg. Peak Area (n=3) | 7760177 | 7461355 | 7670492 | 1.762 |
| | %RSD | 0.356 | 0.037 | 0.419 | |
| LDA | Avg. Peak Area (n=3) | 1904027 | 1925892 | 1880793 | 1.102 |
| | %RSD | 0.476 | 0.418 | 0.493 | |
| LDO | Avg. Peak Area (n=3) | 2615664 | 2662741 | 2556921 | 1.936 |
| | %RSD | 1.302 | 0.367 | 0.906 | |

**Effect of variation in mobile phase flow rate:** Flow rate of mobile phase was changed to 0.6 mL/min and 1.0 mL/min as compared to optimized flow rate of 0.8 mL/min used in the method (0.2 mL variation).

**Table 6.62. Robustness: Effect of Mobile Phase Flow Rate**

| Drug/ Degradant | Mobile Phase Flow Rate | 0.6 mL/min | 0.8 mL/min (optimized) | 1.0 mL/min | % RSD |
|---|---|---|---|---|---|
| LEV | Avg. Peak Area (n=3) | 7418804 | 7461355 | 7463831 | 0.308 |
| | %RSD | 0.496 | 0.037 | 0.386 | |
| LDA | Avg. Peak Area (n=3) | 1935319 | 1925893 | 1935899 | 0.424 |
| | %RSD | 0.371 | 0.418 | 0.390 | |
| LDO | Avg. Peak Area (n=3) | 2648580 | 2662741 | 2653738 | 0.554 |
| | %RSD | 0.936 | 0.367 | 0.393 | |

**Assay of Marketed Formulation:**

The proposed HPLC method was extended for the determination of LEV in tablets. The tablet used was Leon 500 (batch no. LB80304) manufactured by Dr. Reddy's Laboratories Ltd. It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 2. Sample concentration of 60 µg/mL was injected into the chromatograph and resulting peak area from chromatogram recorded.

Average area (6 injections) for assay solution was calculated to be 7377166.

The concentration of sample LEV sample was calculated to be 60.674 µg/mL and assay results found to be complying 101.12 % with label claim (I.P. limits 90-110%). The system suitability and validation results are summarized in Table 6.63.

**Table 6.63. System Suitability and Validation Results for Method 2**

| System Suitability Parameters | LEV | LDO (Degradant 1) | LDA (Degradant 2) |
|---|---|---|---|
| N | 3419 | 3497 | 3291 |
| Tailing factor (Pk sym) | 1.502 | 1.419 | 1.368 |
| RSD of area | 0.577 | 1.068 | 0.546 |
| $R_t$ (min) | 4.118 | 5.041 | 3.545 |
| RSD of $R_t$ (%) | 0.434 | 0.280 | 0.284 |
| $R_s$ with adjacent peak | 2.932 | 4.565 | 2.169 |
| Linearity | y = 121479x + 6584.9<br>$R^2$ =0.9992 | NA | NA |
| LOD(µg/mL) | 5.92 | NA | NA |
| LOQ(µg/mL) | 19.75 | NA | NA |
| Precision (RSD < 2%) | Complies | Complies | Complies |
| Assay of marketed formulation<br>(Limits 90-110%) | 101.12% | NA | NA |
| Accuracy | 98-103% | NA | NA |
| Robustness (RSD < 2%) | Complies | Complies | Complies |

### 6.2.3 Method 3

For a LC-MS compatible mobile phase system for LEV, acetonitrile (ACN), 0.1% triethylamine (TEA) and formic acid was chosen for several trials with various other mobile phases.

#### 6.2.3.1 Trials

**Table 6.64. Trials conducted to find suitable LC-MS compatible HPLC method for LEV and its degradants**

| Trial No. | Experimental Conditions (Mobile Phase & Flow Rate) | Observations | Chromatogram |
|---|---|---|---|
| 1 | ACN: acetate buffer, 20mM, pH 3.0, (20:80 and 30:70), 1.0ml/min | Reasonably good for LDA at 30:70 and 20:80, but not good separation for LDO | Fig. 6.16, 6.17 & 6.18 |
| 2 | ACN: acetate buffer, 20mM, pH 6.0 (30 :70, 35:65), 0.8 &1.0ml/min | Separation not good, peak shapes show splits and shoulders | Fig. 6.19, 6.20, 6.21 & 6.22 |
| 3 | ACN: acetate buffer, 30mM, pH 3.0 30 :70), 1.0ml/min | No separation for LDO | Fig. 6.23 |
| 4 | ACN: acetate buffer, 50mM, pH 3.0 (30 :70), 1.0ml/min | No separation for LDO | Fig. 6.24 |
| 5 | ACN: 0.1% formic acid (pH not adjusted); 25:75 | Good separation at 25:75, but high tailing | Fig. 6.25, 6.26 |
| 6 | ACN: 0.1% TEA, pH adjusted with Formic acid (17.5:82.5)<br>(pH 3.0 & 3.5) | Good separation, tailing decreased, but needs further improvement | Fig. 6.27, 6.28 |
| 7 | 15:85 of ACN: 0.1% TEA, pH adjusted to 3.0 with Formic acid | Method 3 | Fig. 6.29 |

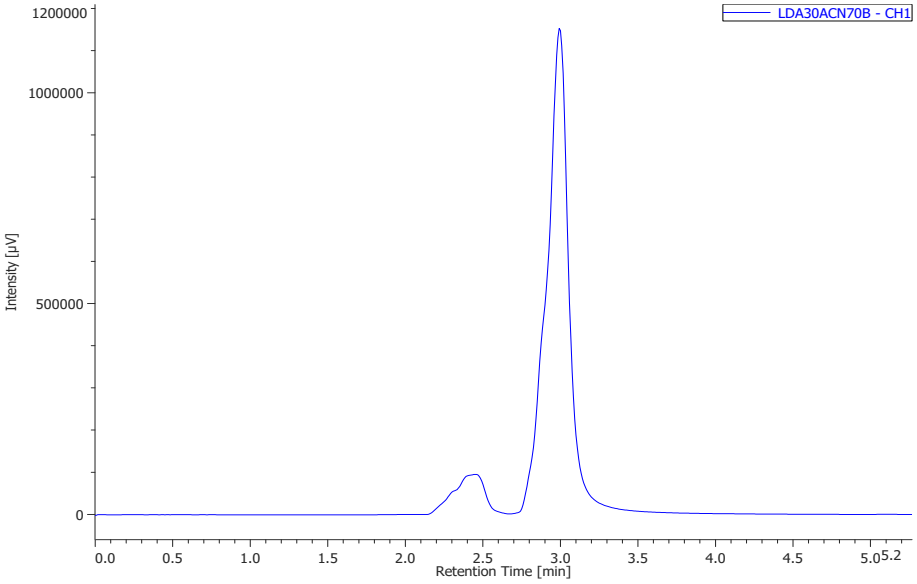



**Fig. 6.16. Chromatogram of LDA, 30:70 ACN and acetate buffer (pH 3.0, 20 mM), 1.0 mL/min**

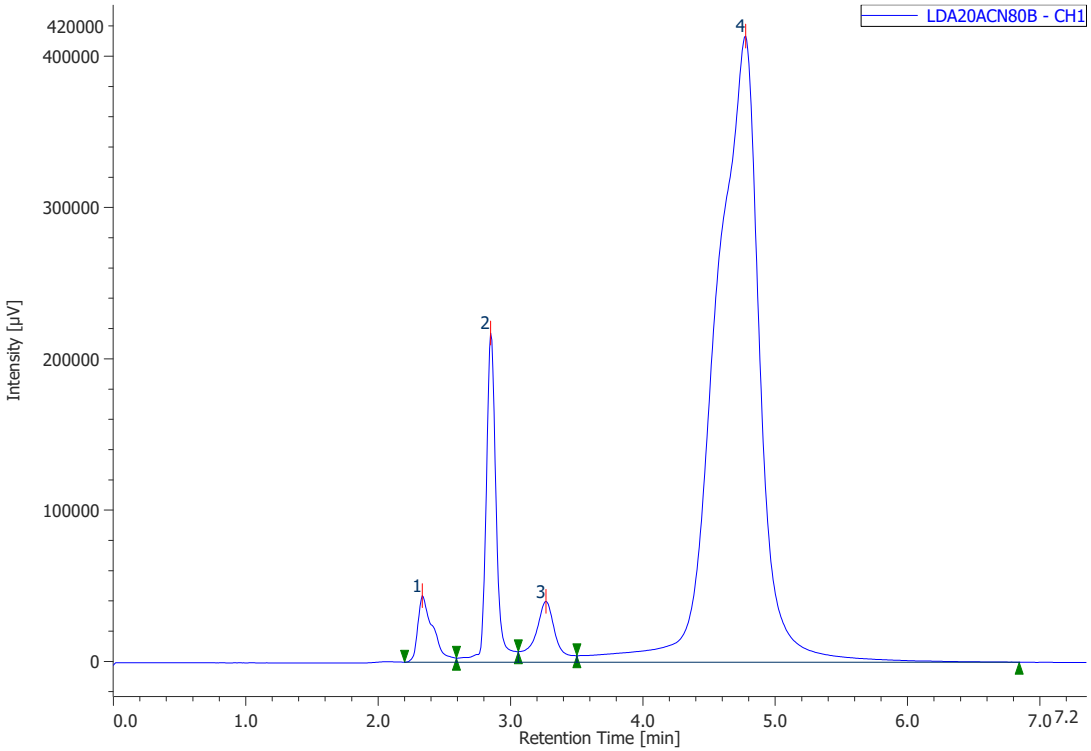



**Fig. 6.17. Chromatogram of LDA, 20:80 ACN and acetate buffer (pH 3.0, 20 mM), 1.0 mL/min**

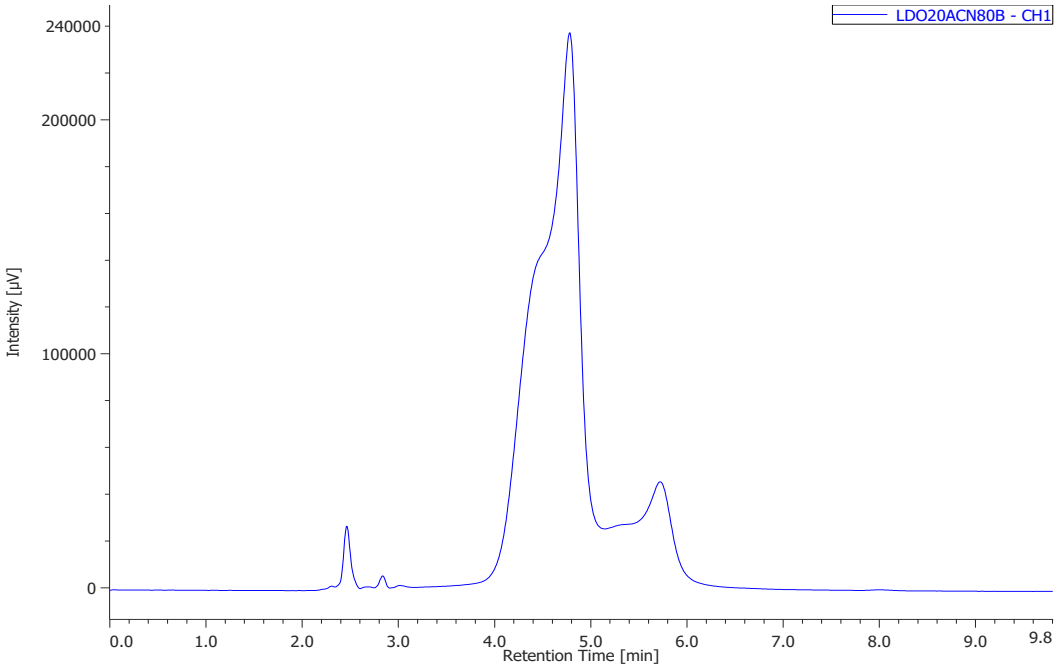



**Fig. 6.18. Chromatogram of LDO, 20:80 ACN and acetate buffer (pH 3.0, 20 mM), 1.0 mL/min**

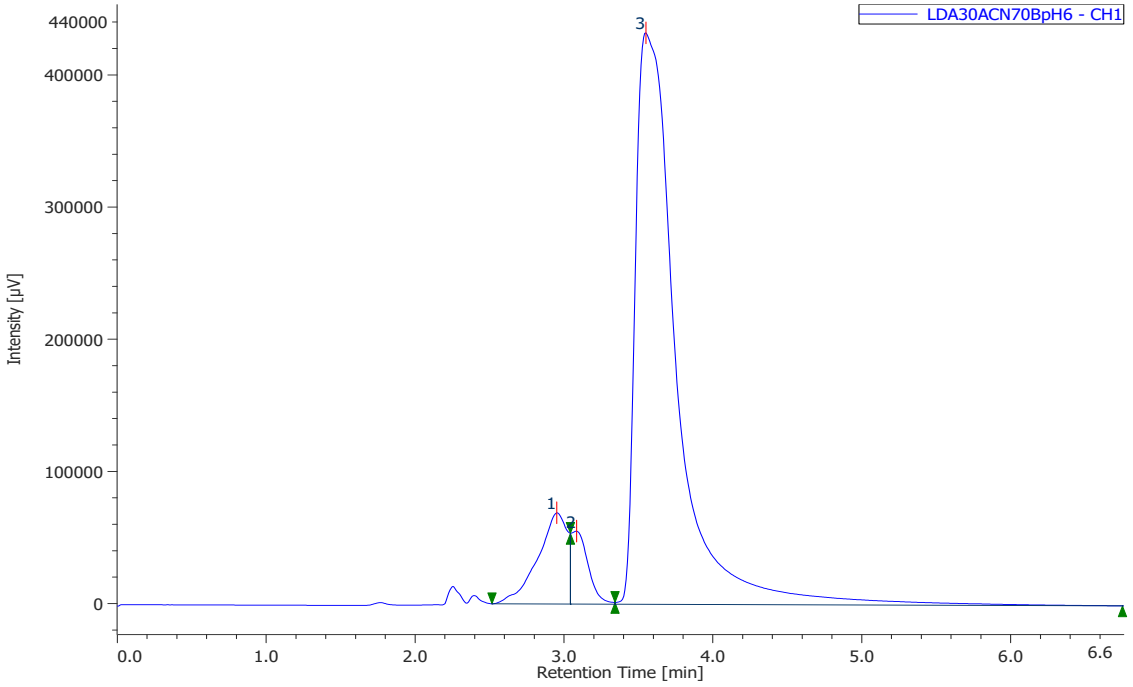



**Fig. 6.19. Chromatogram of LDA, 30:70 ACN and acetate buffer (pH 6.0, 20 mM), 1.0 mL/min**

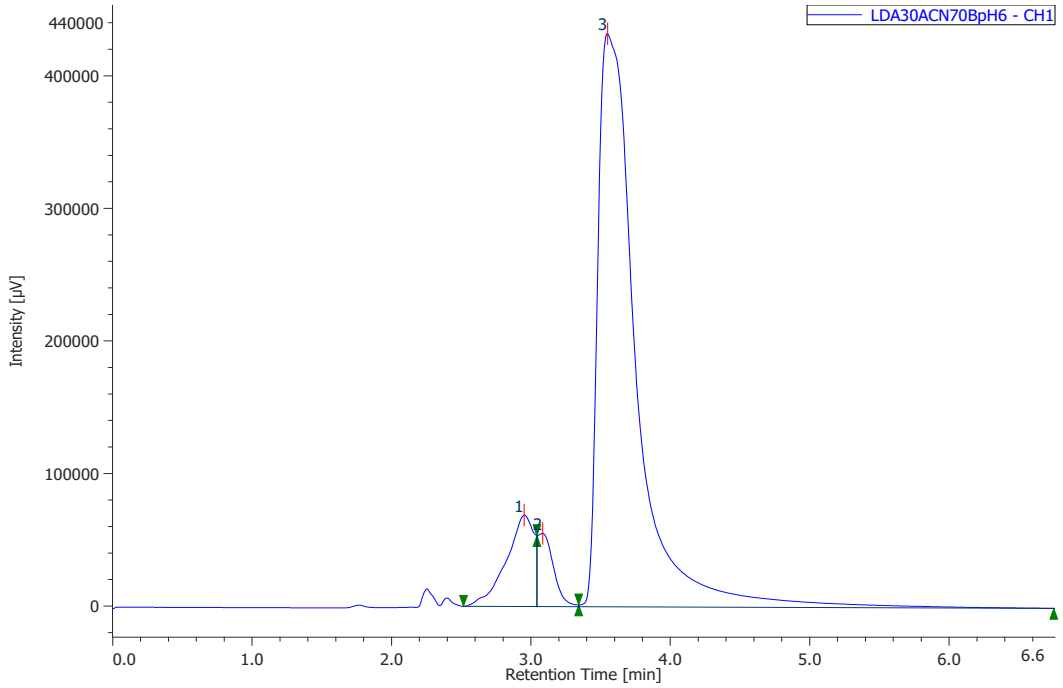



**Fig. 6.20. Chromatogram of LDA, 35:65 ACN and acetate buffer (pH 6.0, 20 mM), 1.0 mL/min**

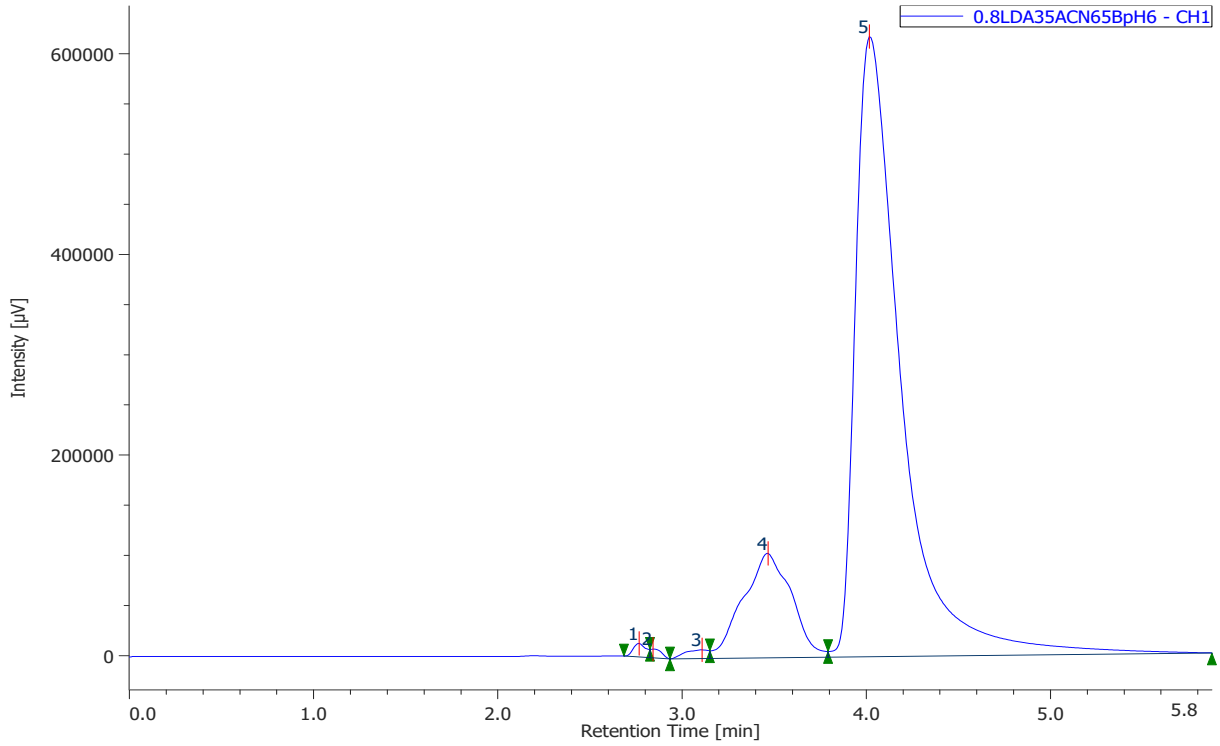



**Fig. 6.21. Chromatogram of LDA, 35:65 ACN and acetate buffer (pH 6.0, 20 mM) 0.8 mL/min**

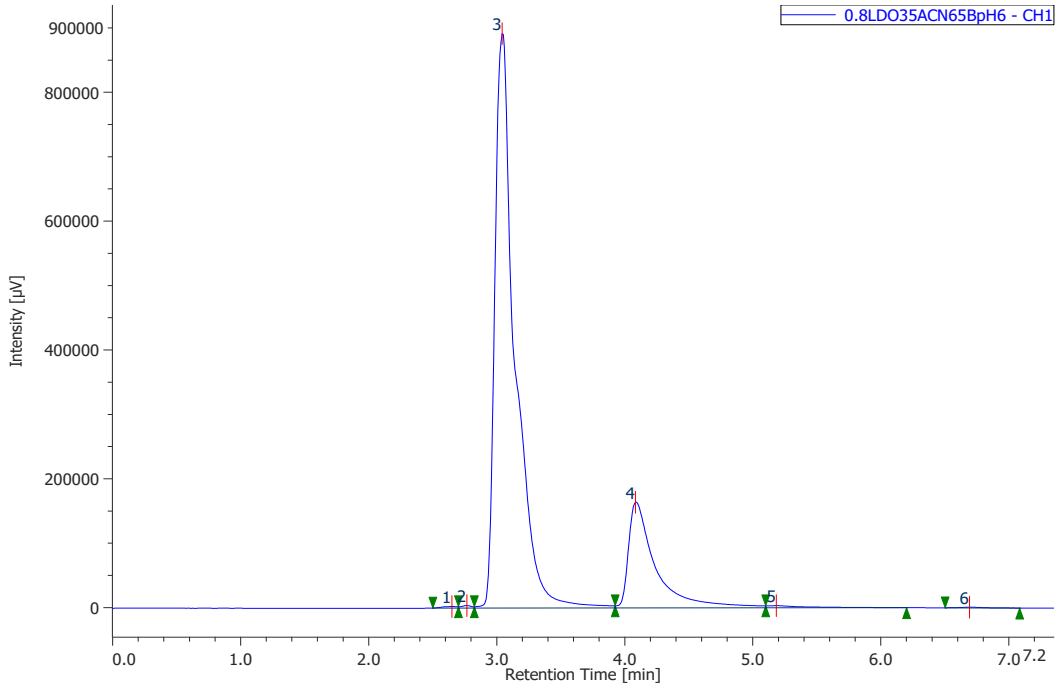



**Fig. 6.22. Chromatogram of LDO, 35:65 ACN and acetate buffer (pH 6.0, 20 mM) 1.0 mL/min**

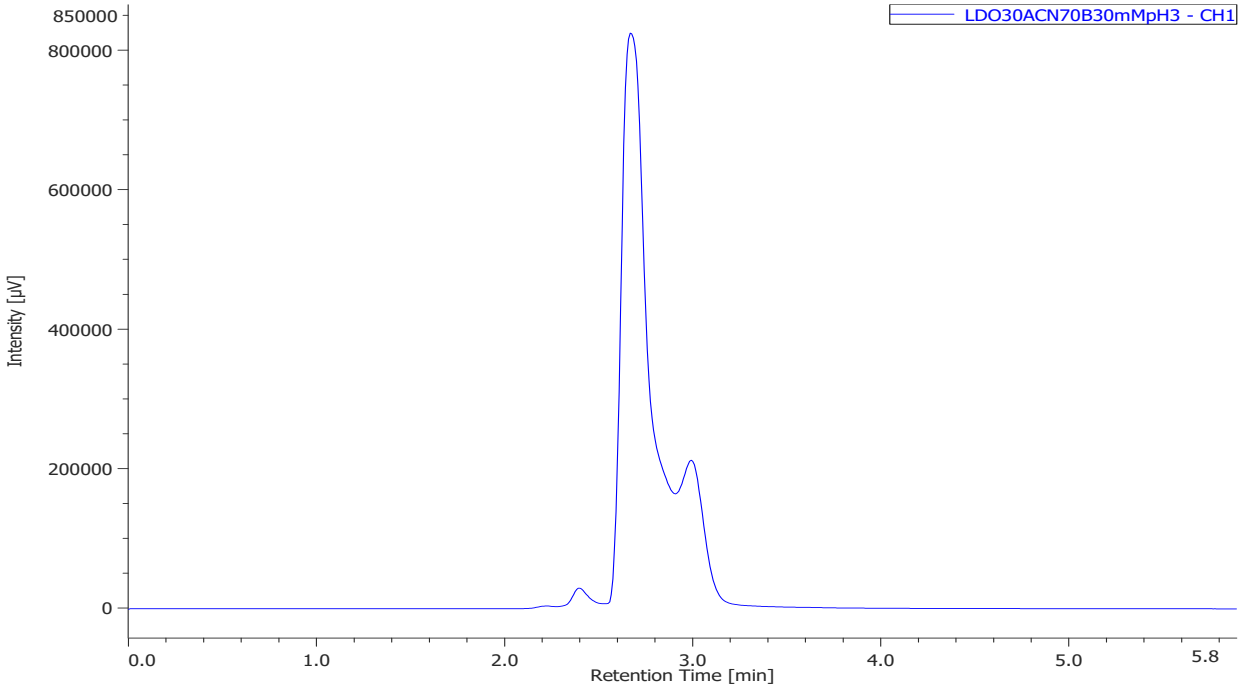



**Fig. 6.23. Chromatogram of LDO, 30:70 ACN and acetate buffer (pH 3.0, 30 mM) 1.0 mL/min**

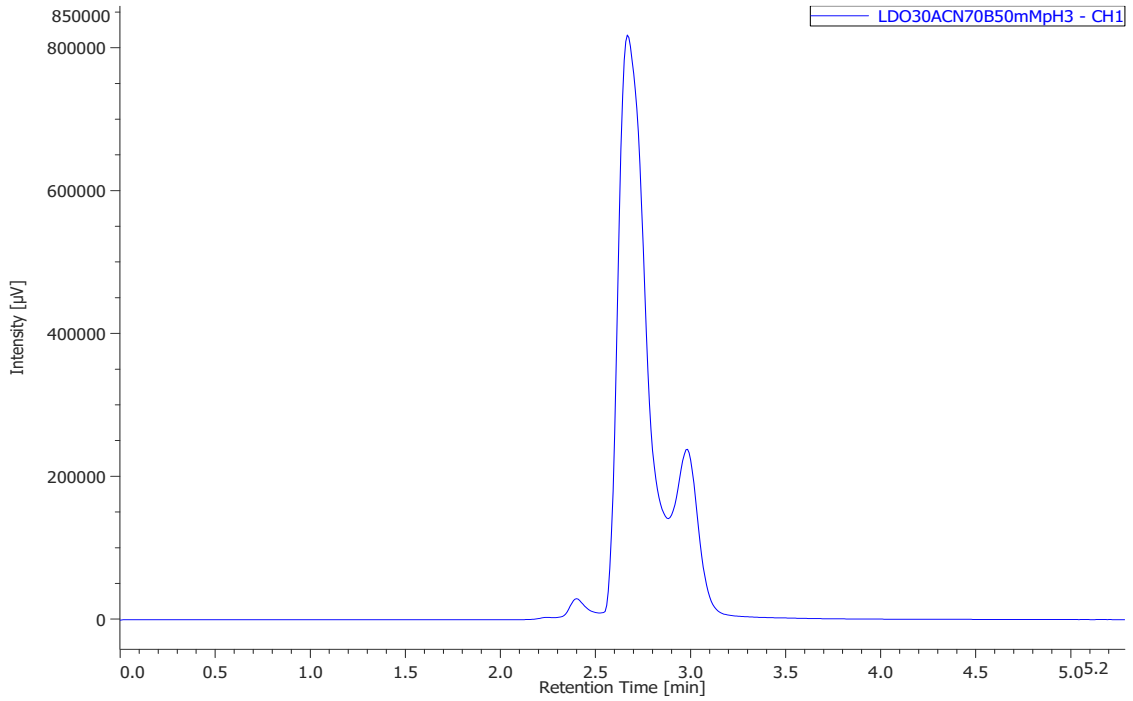



**Fig. 6.24. Chromatogram of LDO, 30:70 ACN and acetate buffer (pH 3.0, 30 mM) 1.0 mL/min**

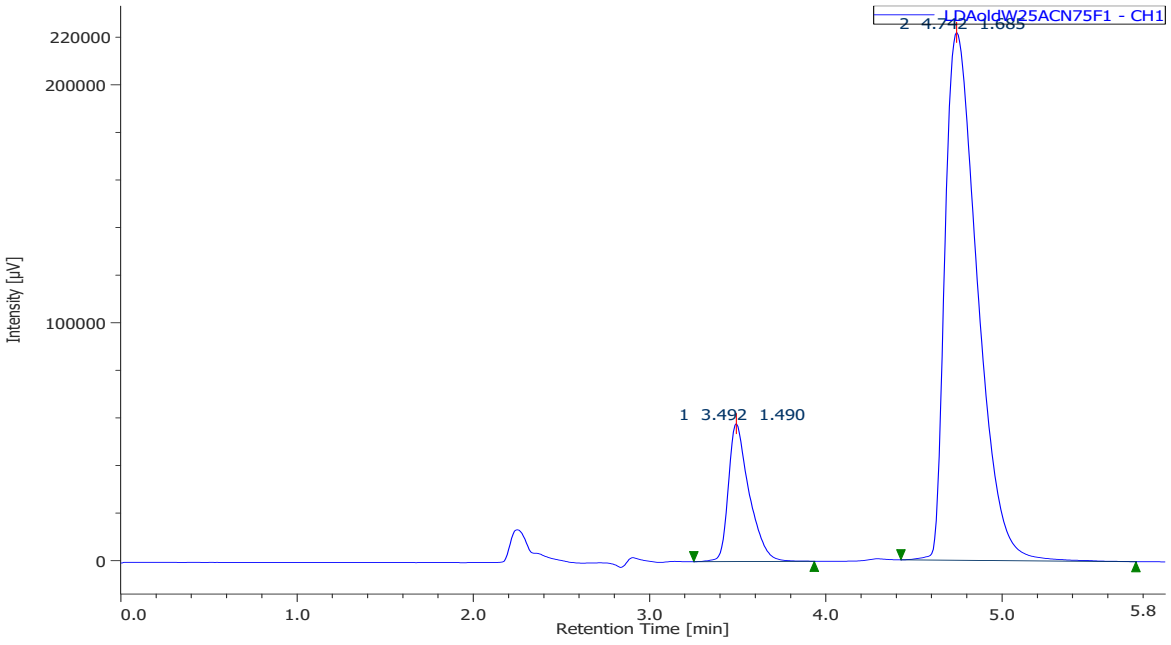



**Fig. 6.25. Chromatogram of LDA, 25:75 ACN: 0.1% formic acid, 1.0 mL/min**

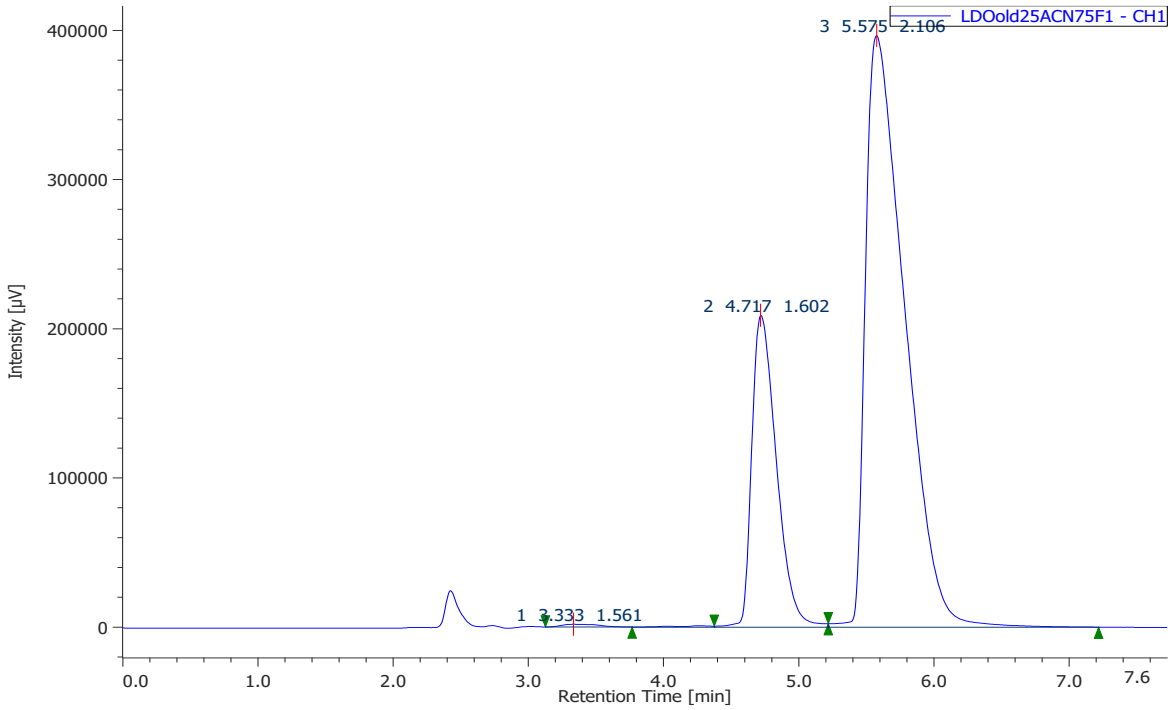



**Fig. 6.26. Chromatogram of LDO, 25:75 ACN: 0.1% formic acid, 1.0 mL/min**

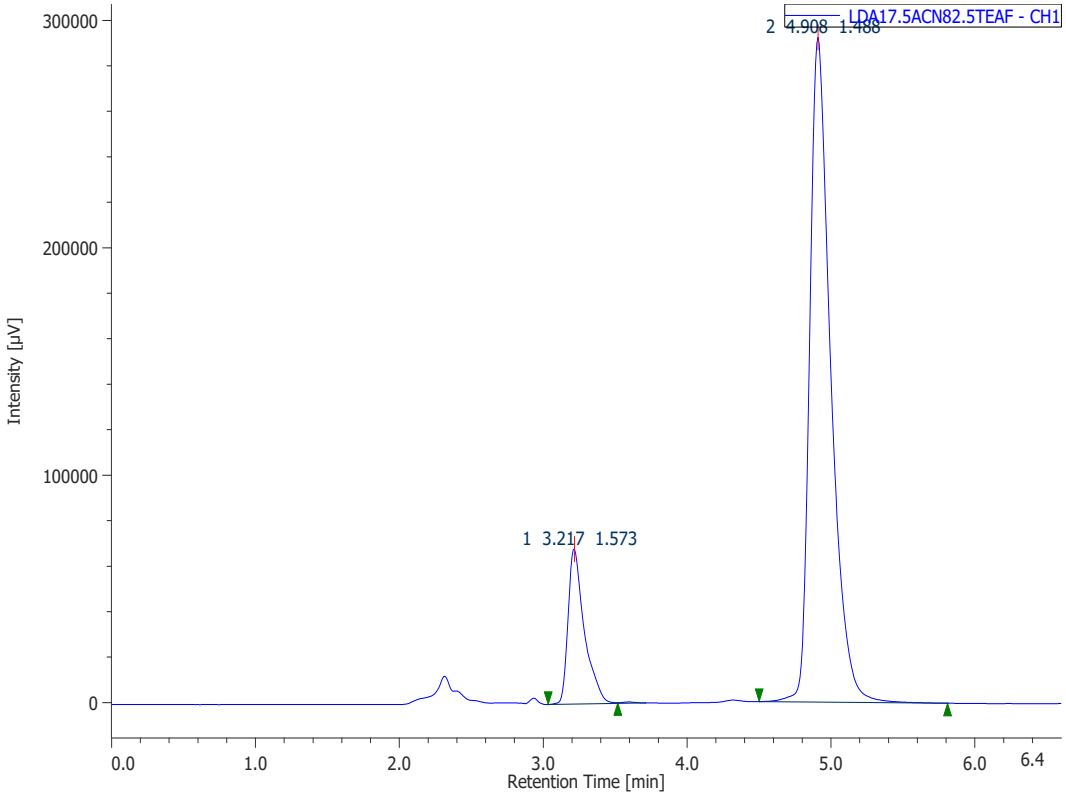



**Fig. 6.27. Chromatogram of LDA, 17.5:82.5 ACN: 0.1% TEA, pH 3.0 with formic acid, 1.0 mL/min**

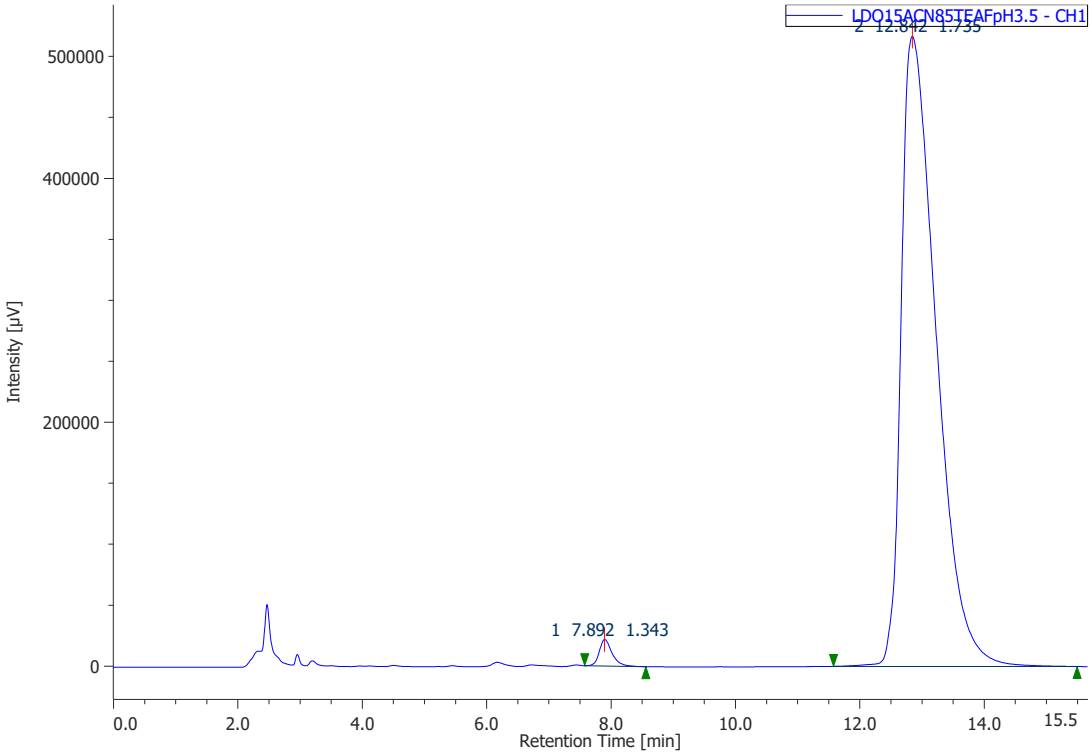



**Fig. 6.28. Chromatogram of LDA, 15:85 ACN: 0.1% TEA, pH 3.5 with formic acid, 1.0 mL/min**

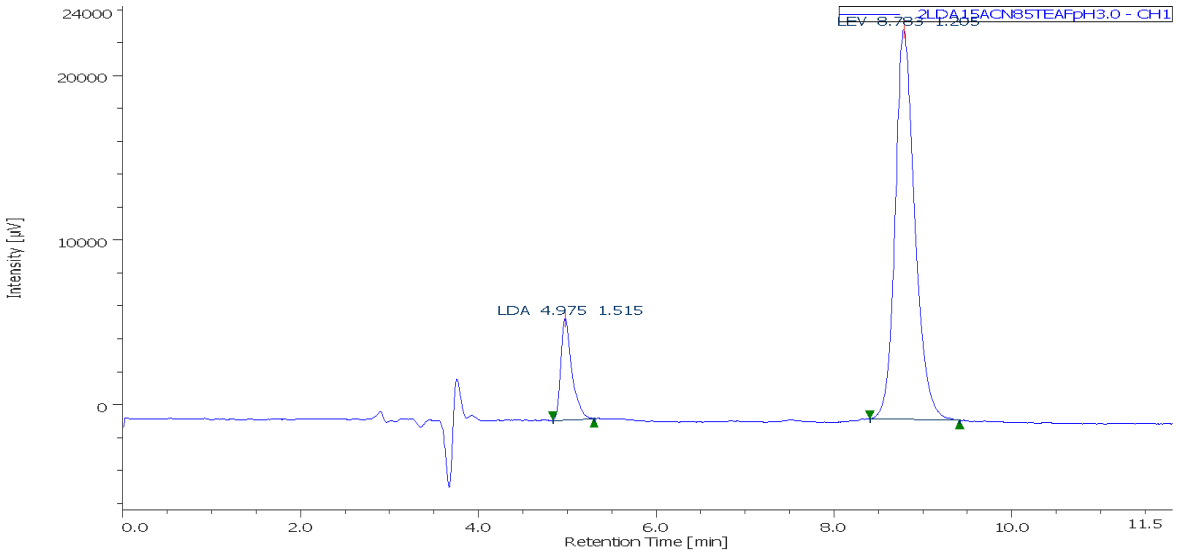



**Fig. 6.29. Chromatogram of LEV sample degraded by acid hydrolysis (LDA) using Method 3**

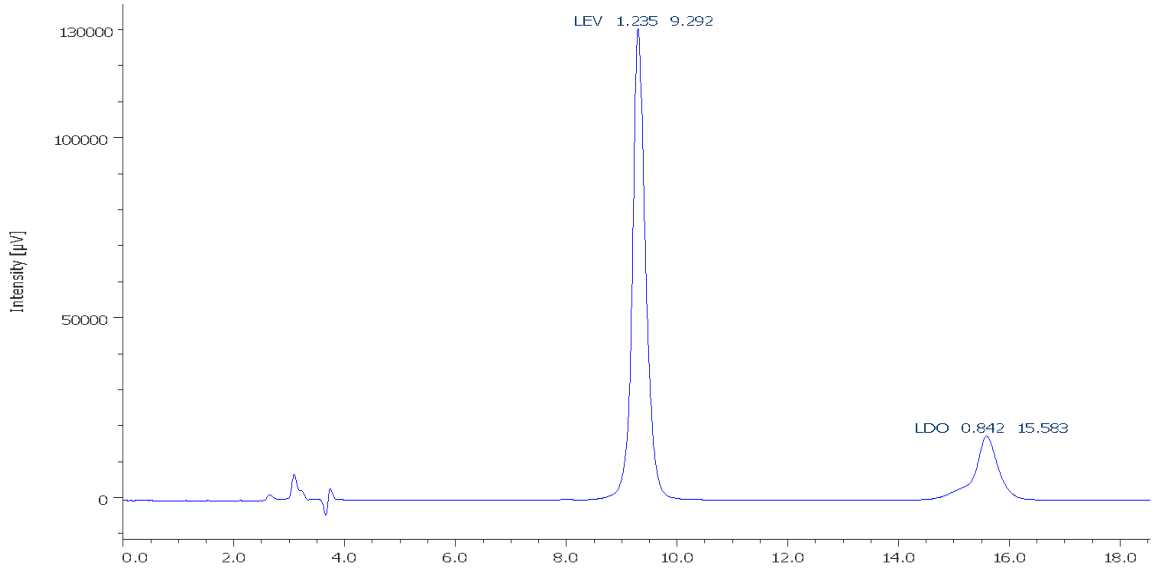



**Fig. 6.30. Chromatogram of LEV sample degraded by oxidation (LDO) using Method 3**

**Table 6.65. Experimental Variables for Proposed Method 3.**

| Method No. | Column | Column Temperature | Mobile Phase | Flow rate (mL/min) | Applicable to |
|---|---|---|---|---|---|
| 3 | C18 | ambient | ACN: 0.1% TEA, adjusted to pH 3.0 with Formic Acid (15:85) | 0.8 | Acid and oxidative degradants of LEV and LCMS compatible |

Method 3 used ACN: 0.1% TEA, adjusted to pH 3.0 with formic acid in proportion of 15:85 at a flow rate of 0.8 mL/min and achieved good resolution between LEV and its degradants. The chromatograms obtained using Developed Method 3 have been presented as Figures 6.29 and 6.30. The corresponding System Suitability and Validation Parameters have been tested and reported in following section.

### 6.2.3.2. System Suitability and Validation of Method 3

The system suitability and validation parameters achieved through Method 3, following procedures as per section 5.3.1.4 (pp 124) have been reported here.

LDA is the degradant produced through acid hydrolysis of LEV and LDO the degradant in oxidized sample of LEV.

**Table 6.66. System Suitability Parameters of Method 3 for LEV and acid hydrolysis degradant LDA**

| System Suitability Parameters | LEV | LDA | LDO |
|---|---|---|---|
| N | 7642.333 | 7593.667 | 7541.000 |
| Tailing factor (Pk. sym) | 1.243 | 1.522 | 0.852 |
| RSD of area (%) | 1.861 | 1.155 | 0.270 |
| $R_t$ | 8.742 | 4.972 | 15.523 |
| RSD of $R_t$ (%) | 0.255 | 0.093 | 0.155 |
| $R_s$ with adjacent peak | ------- | 12.000 | 10.945 |

**Precision Study for Analysis of LEV in presence of degradant:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of the dilutions (conc. 50 µg/mL) made from tablet extract (as per procedure given under section 5.3.1.4.9, pp 131) on two different days.

Acceptance criteria: % RSD should be <2%.

**Table 6.67. Precision Study Data of LEV (Conc. 50 µg/mL)**

| Day 1 | | | Day 2 | | |
|---|---|---|---|---|---|
| Sr. No. | Peak Area | Conc (µg/mL) | Sr. No. | Peak Area | Conc (µg/mL) |
| 1 | 1047997 | 50.821 | 1 | 1048134 | 50.828 |
| 2 | 1048332 | 50.837 | 2 | 1049412 | 50.888 |
| 3 | 1050121 | 50.922 | 3 | 1046785 | 50.764 |
| 4 | 1049736 | 50.904 | 4 | 1050163 | 50.924 |
| 5 | 1047929 | 50.818 | 5 | 1048145 | 50.828 |
| 6 | 1046546 | 50.752 | 6 | 1047815 | 50.812 |
| Mean | 1048444 | 50.842 | Mean | 1048409 | 50.841 |
| SD | 1308.554 | 0.057 | SD | 1201.504 | 0.052 |
| %RSD | 0.125 | 0.112 | %RSD | 0.115 | 0.102 |
| Inter-day Precision | | | | | |
| Mean | 1048426 | 50.84142 | | | |
| SD | 1197.847 | 0.056827 | | | |
| %RSD | 0.114252 | 0.111772 | | | |

**Linearity:** The response was linear over concentration range of 10-120 µg/mL as presented in Table 6.68, with $R^2$ value of 0.999. The linearity graph is shown as Fig. 6.31.

**Table 6.68. Linearity: Peak areas and Concentration of LEV**

| Conc µg/mL | Peak Area | | | | SD | %RSD |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | avg | | |
| 10 | 185726 | 184968 | 183421 | 184705.0 | 1174.791 | 0.636 |
| 20 | 375271 | 379828 | 370889 | 375329.3 | 4469.785 | 1.191 |
| 30 | 602349 | 610167 | 608110 | 606875.3 | 4052.602 | 0.668 |
| 50 | 1055937 | 1049651 | 1037115 | 1047568.0 | 9582.386 | 0.915 |
| 80 | 1664197 | 1669569 | 1648040 | 1660602.0 | 11205.690 | 0.675 |
| 100 | 2084065 | 2095999 | 2077078 | 2085714.0 | 9567.678 | 0.459 |
| 120 | 2487495 | 2504409 | 2513891 | 2501932.0 | 13371.240 | 0.534 |

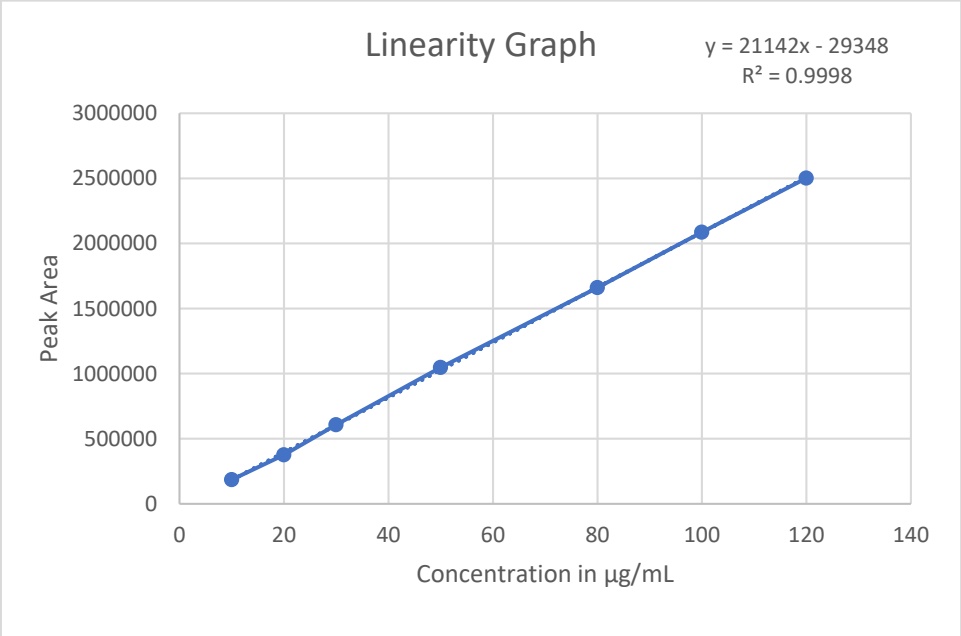



**Fig. 6.31. Linearity Graph for LEV (Method 3)**

Limit of Detection and Limit of Quantitation were calculated and found to be:

Limit of Detection, LOD (calculated) =1.287 µg/mL

Limit of Quantitation, LOQ (calculated) =4.290 µg/m

**Accuracy:**

Accuracy (% Recovery) was assessed in triplicate, at three different levels of concentrations ranging from 80, 100 and 120% of the target concentration of active ingredient, by spiking a known amount of each of the standard to a sample of known concentration (prepared from tablet extract) and then calculating the recovery and RSD (%) for each concentration as per procedure given in section 5.3.1.4.7 (pp 129).

**Table 6.69. Results of Accuracy of Method 3 for LEV**

| Level of addition (std) (%) | Amount of std added (µg/mL) | Avg. Peak Area | Conc (µg/mL) | Assay | Recovery | % recovery |
|---|---|---|---|---|---|---|
| 80 | 40 | 1865425 | 89.600 | 49.571 | 40.029 | 100.073 |
| 100 | 50 | 2085723 | 100.051 | 49.571 | 50.480 | 100.960 |
| 120 | 60 | 2298078 | 110.126 | 49.571 | 60.555 | 100.924 |

**Robustness**: The effect of changes in mobile phase proportion, pH and flow rate on retention time, peak area, number of theoretical plates and Tailing Factor was studied.

**Effect of mobile phase proportion**: Mobile phase ratios 13:87 and 17:83 of methanol: buffer as compared to optimized ratio of 15:85 used in the method.

**Table 6.70. Robustness: Effect of Mobile Phase Ratio**

| Drug/ Degradant | Mobile Phase Ratio | 13:87 | 15:85 (optimized) | 17:83 | % RSD |
|---|---|---|---|---|---|
| LEV | Avg. Peak Area (n=3) | 404809 | 391593 | 396258 | 1.883 |
| | %RSD | 0.883 | 0.705 | 1.790 | |
| LDA | Avg. Peak Area (n=3) | 59254 | 56896 | 57338 | 1.880 |
| | %RSD | 1.367 | 0.893 | 1.193 | |

**Effect of variation in pH**: pH of aqueous phase was adjusted to 2.8 and 3.2 as compared to optimized pH of 3.0 used in the method (0.2 units variation)

**Table 6.71. Robustness: Effect of pH**

| Drug/ Degradant | pH | 2.8 | 3.0 (optimized) | 3.2 | % RSD |
|---|---|---|---|---|---|
| LEV | Avg. Peak Area (n=3) | 392210 | 391593 | 392343 | 1.56 |
| | %RSD | 0.188 | 0.705 | 0.328 | |
| LDA | Avg. Peak Area (n=3) | 56611 | 56896 | 56999 | 1.11 |
| | %RSD | 0.927 | 0.893 | 0.657 | |

**Effect of variation in mobile phase flow rate**: Flow rate of mobile phase was changed to 0.6 mL/min and 1.0 mL/min as compared to optimized flow rate of 0.8 mL/min used in the method (0.2 mL variation).

**Table 6.72. Robustness: Effect of Mobile Phase Flow Rate**

| Drug/ Degradant | Mobile Phase Flow Rate | 0.6 mL/min | 0.8 mL/min (optimized) | 1.0 mL/min | % RSD |
|---|---|---|---|---|---|
| LEV | Avg. Peak Area (n=3) | 39669 | 391593 | 399676 | 1.704 |
| | %RSD | 0.980 | 0.705 | 1.871 | |
| LDA | Avg. Peak Area (n=3) | 57380 | 56896 | 56902 | 0.828 |
| | %RSD | 0.383 | 0.893 | 0.310 | |

**Assay of Marketed Formulation:**

The proposed HPLC method was extended for the determination of LEV in tablets. The tablet used was Leon 500 (batch no. LB80304) manufactured by Dr. Reddy's Laboratories Ltd. It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 3. Sample concentration of 50 µg/mL was injected into the chromatograph and resulting peak area from chromatogram recorded.

Average area (6 injections) for assay solution was calculated to be 1048444.

The concentration of sample OFL sample was calculated to be 49.571 µg/mL and assay results found to be complying 98.71 % with label claim (I.P. limits 90-110%).

The system suitability and validation results are summarised in Table 6.73.

**Table 6.73. System Suitability and Validation Parameters using proposed Method 3 for LEV and degradant LDA using mobile phase with ACN: 0.1% TEA adjusted to pH 3.0 with formic acid in the ratio 15:85, with flow rate 0.8 mL/min**

| System Suitability Parameters | LEV | LDA (Degradant) |
|---|---|---|
| N | 7642.333 | 7593.667 |
| Tailing factor (Pk.sym) | 1.243 | 1.522 |
| % RSD of area (<2%) | 1.861 | 1.155 |
| $R_t$ (min) | 8.742 | 4.972 |
| % RSD of $R_t$(<2%) | 0.255 | 0.093 |
| $R_s$ with adjacent peak | NA | 12.000 |
| Linearity | y = 21142x – 29348<br>$R^2$ = 0.9998 | NA |
| LOD(µg/mL) | 1.287 | NA |
| LOQ(µg/mL) | 4.290 | NA |
| Precision (RSD < 2%) | Complies | NA |
| Assay of marketed formulation (Limits 90-110%) | 98.71% | NA |
| Accuracy | 100-101% | NA |
| Robustness (RSD < 2%) | Complies | Complies |

### 6.2.4 Method 4

For a LC-MS compatible mobile phase system for CIP, acetonitrile (ACN), 0.1% triethylamine (TEA) and formic acid was chosen for several trials with various other mobile phases.

#### 6.2.4.1 Trials

**Table 6.74. Trials conducted to find suitable LC-MS compatible HPLC method for CIP and its degradants**

| Trial No. | Experimental Conditions | Observations | Chromatogram |
|---|---|---|---|
| 1 | ACN and 0.1% TEA, pH 3.0 (20:80; 0.8ml/min) | Peak of degradant not well-separated from CIP | Fig. 6.32 |
| 2 | ACN and 0.1% TEA, pH 3.0 (15:85; 0.8ml/min) | Peaks of degradant seen<br>Good N but low Rs | Fig. 6.33 |
| 3 | ACN and 0.1% TEA, pH 3.0 (13:87, 0.8ml/min) | N above 3000, PS 1.1 to1.4,<br>Rs good | Fig. 6.34 |
| 4 | ACN and 0.1% TEA, pH 3.0 (13:87, 1.0ml/min) | N above 2000, PS satisfactory,<br>Rs good, run time long | Fig. 6.35 |
| 5 | ACN and 0.1% TEA, pH 3.0 (13:87, 1.5ml/min) | N above 2000, PS satisfactory,<br>Rs good, run time 12min | Fig. 6.36, 6.37 |

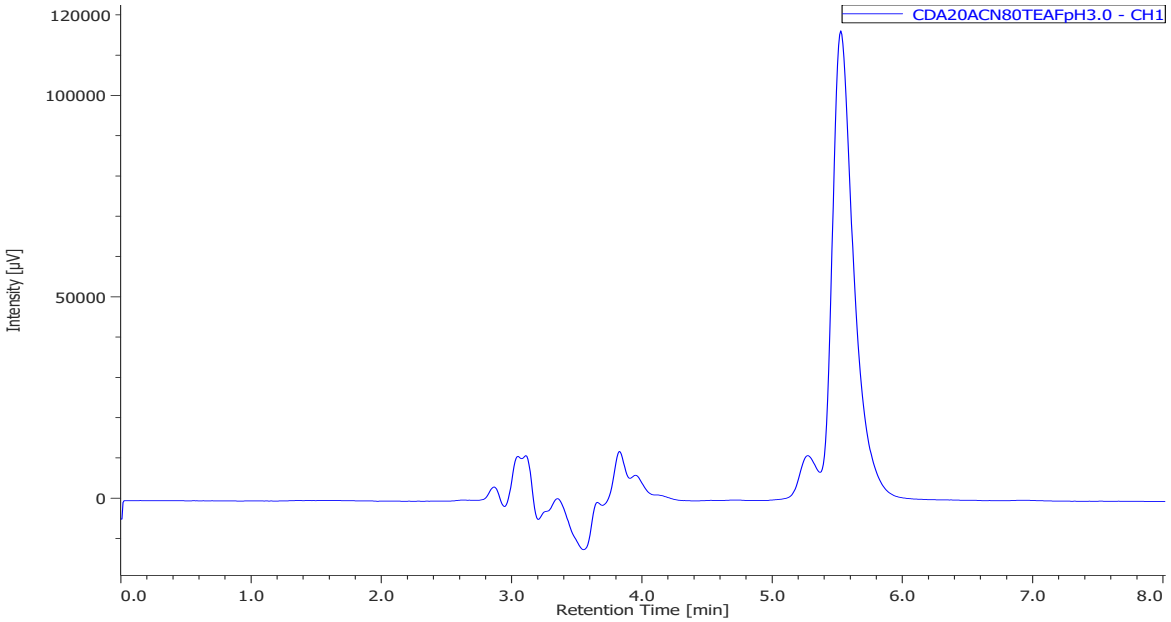



**Fig. 6.32. Chromatogram of CDA with mobile phase 20:80 of ACN: 0.1% TEA, pH adjusted to 3.0 with Formic acid, flow rate of 0.8 mL/min**

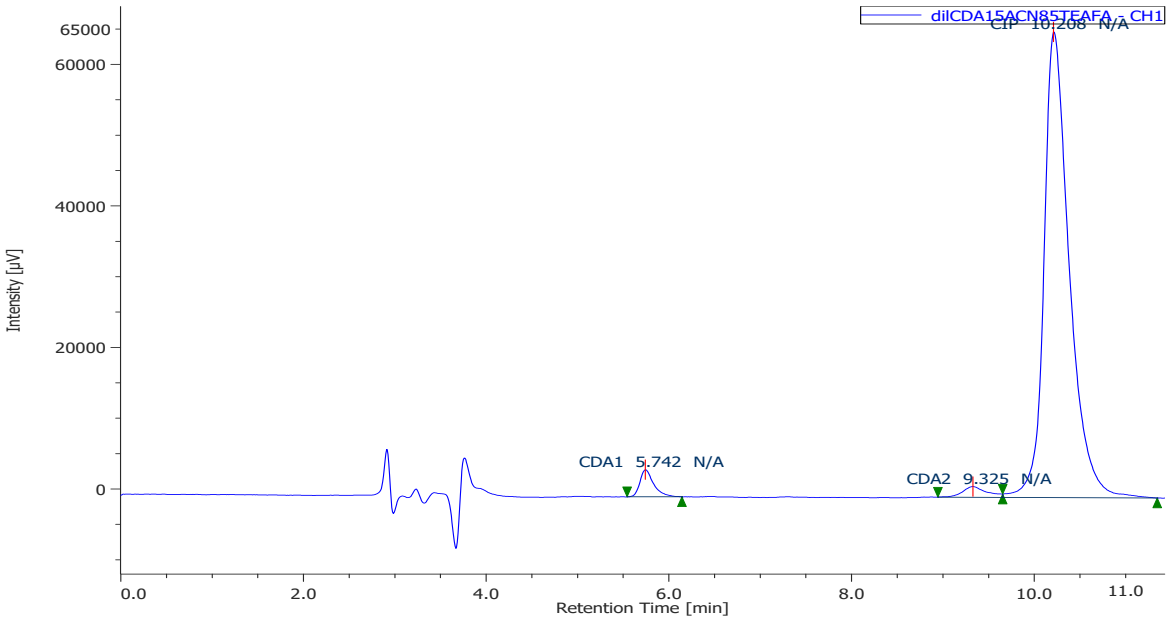



**Fig. 6.33. Chromatogram of CDA with mobile phase 15:85 of ACN: 0.1% TEA, pH adjusted to 3.0 with Formic acid, flow rate of 0.8 mL/min**

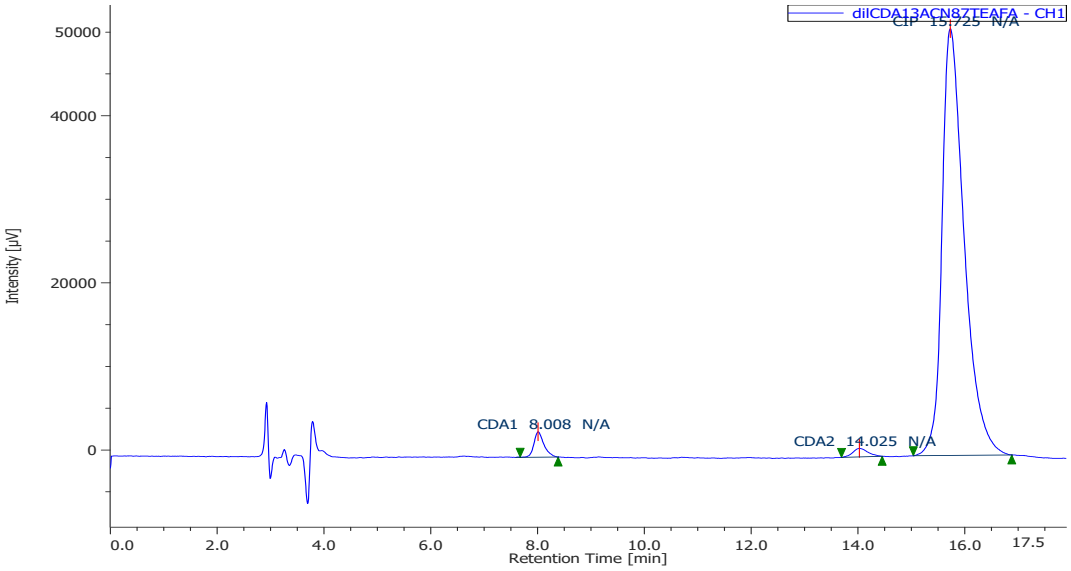



**Fig. 6.34. Chromatogram of CDA with mobile phase 13:87 of ACN: 0.1% TEA, pH adjusted to 3.0 with Formic acid, flow rate of 0.8 mL/min**

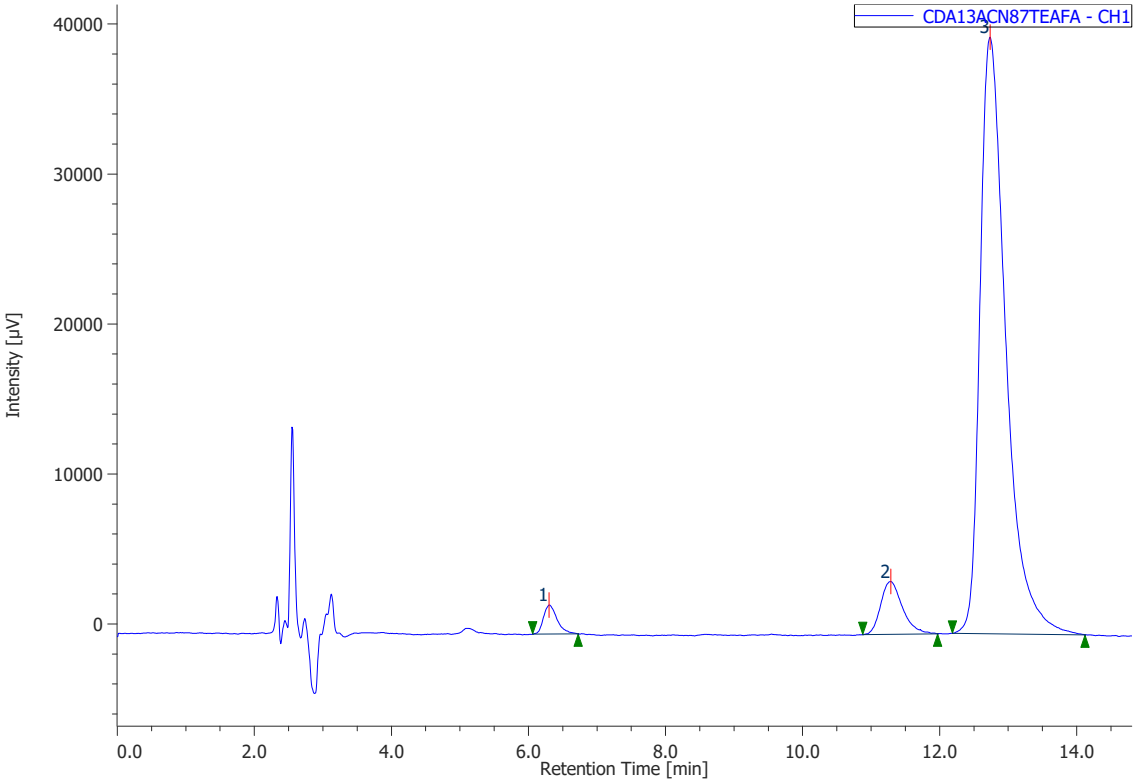



**Fig. 6.35. Chromatogram of CDA with mobile phase 13:87 of ACN: 0.1% TEA, pH adjusted to 3.0 with Formic acid, flow rate of 1.0 mL/min**

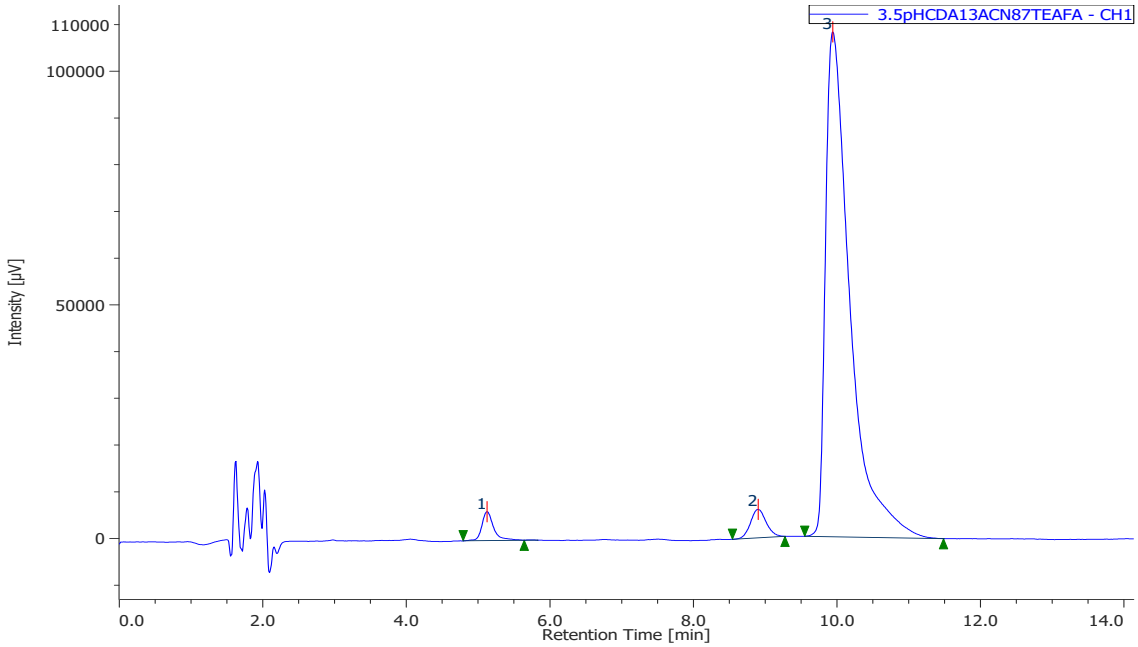



**Fig. 6.36. Chromatogram of CDA with mobile phase 13:87 of ACN: 0.1% TEA, pH adjusted to 3.0 with Formic acid, flow rate of 1.5 mL/min**

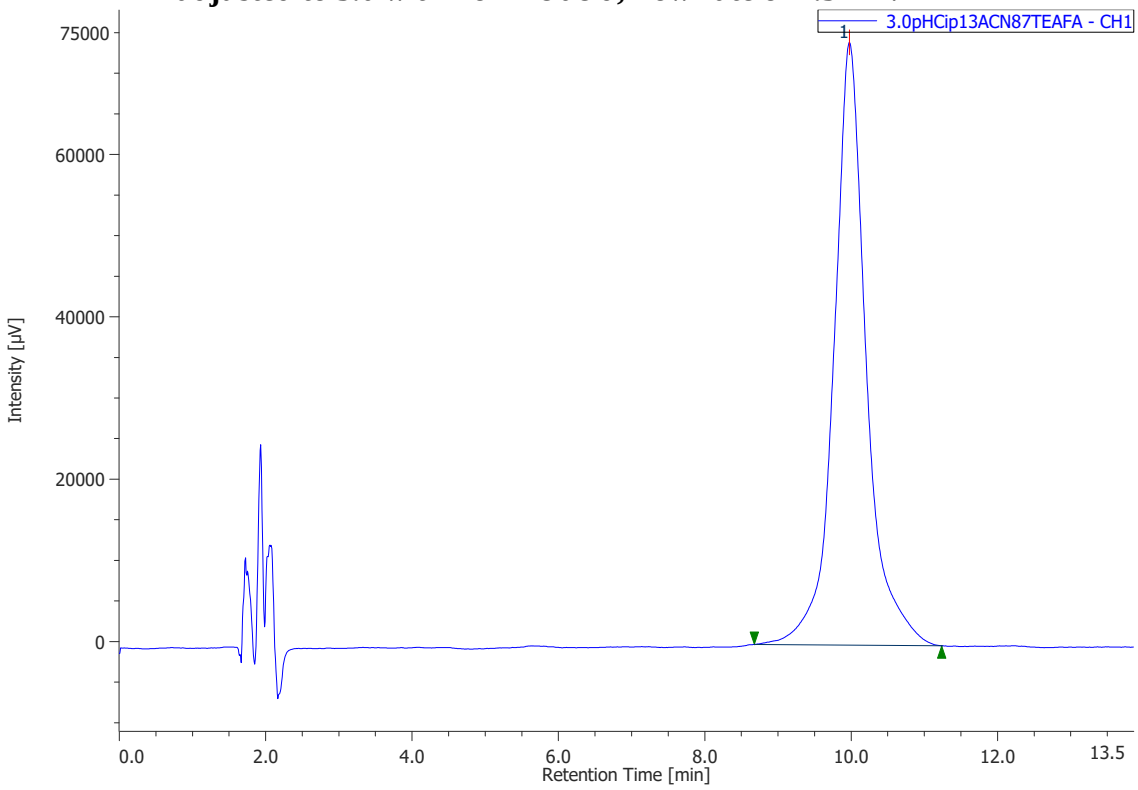



**Fig. 6.37. Chromatogram of CIP with mobile phase 13:87 of ACN: 0.1% TEA, pH adjusted to 3.0 with Formic acid, flow rate of 1.5 mL/min**

**Table 6.75. Experimental Variables for Proposed Method 4.**

| Method No. | Column | Column Temperature | Mobile Phase | Flow rate (mL/min) | Applicable to |
|---|---|---|---|---|---|
| 4 | C18 | ambient | ACN: 0.1% TEA, adjusted to pH 3.0 with Formic Acid (13:87) | 1.5 | Acid hydrolysis degradants of CIP and LCMS compatible |

Method 4 found suitable for application to CIP analysis used a mobile phase having same components, namely, ACN and 0.1% TEA adjusted to pH 3.0 with formic acid in a slightly different proportion of 13:87, but at a higher flow rate of 1.5 mL/min to keep the run time shorter.

### 6.2.4.2. System Suitability and Validation of Method 4

The system suitability and validation parameters achieved through Method 4, following procedures as per section 5.3.1.4 (pp 124) have been reported here.

CDA1 and CDA2 are the degradants produced through acid hydrolysis of CIP.

**Table 6.76. System Suitability Parameters of Method 4 for CIP and acid hydrolysis degradants CDA and CDA2**

| System Suitability Parameters | CDA1 | CDA2 | CIP |
|---|---|---|---|
| N | 5713 | 7744 | 2497 |
| Tailing factor (Pk sym) | 1.469 | 1.109 | 1.114 |
| RSD of area | 0.088 | 0.026 | 0.001 |
| $R_t$ | 5.125 | 8.900 | 9.977 |
| RSD of $R_t$ (%) | 0 | 0.009 | 0.036 |
| $R_s$ with adjacent peak | 11.115 | 2.063 | -------- |

**Precision Study for Analysis of CIP in presence of degradants:**

Repeatability and intermediate precision studies were performed by injecting six replicate injections of dilutions made from tablet extract (concentration 50 µg/mL) prepared as per procedure given under section 5.3.1.4.9 (pp 131), on two different days.

Acceptance criteria: % RSD should be <2%.

**Table 6.77. Precision Study Data of CIP (concentration 50 µg/mL)**

| Day 1 | | | Day 2 | | |
|---|---|---|---|---|---|
| Sr. No. | Peak Area | Conc (µg/mL) | Sr. No. | Peak Area | Conc (µg/mL) |
| 1 | 5689532 | 50.696 | 1 | 5715589 | 50.936 |
| 2 | 5714628 | 50.927 | 2 | 5705266 | 50.841 |
| 3 | 5699522 | 50.788 | 3 | 5711145 | 50.895 |
| 4 | 5687784 | 50.680 | 4 | 5707892 | 50.865 |
| 5 | 5701586 | 50.807 | 5 | 5681678 | 50.623 |
| 6 | 5674412 | 50.557 | 6 | 5697121 | 50.766 |
| Mean | 5694577 | 50.742 | Mean | 5703115 | 50.821 |
| SD | 13811.921 | 0.127 | SD | 12190.170 | 0.112 |
| %RSD | 0.243 | 0.250 | %RSD | 0.214 | 0.221 |
| Inter-day Precision | | | | | |
| Mean | 5698846 | 50.78146 | | | |
| SD | 13196.18 | 0.121436 | | | |
| %RSD | 0.231559 | 0.239134 | | | |

**Linearity:** The response was linear over concentration range of 10-120 µg/mL as presented in Table 6.78, with $R^2$ value of 0.999. Linearity graph is shown in Fig. 6.38.

**Table 6.78. Linearity: Peak areas and Concentration of CIP**

| Conc µg/mL | Peak Area | | | | SD | %RSD |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 10 | 1145789 | 1139046 | 1143555 | 1142797 | 2804.558 | 0.245 |
| 20 | 2285337 | 2285466 | 2285578 | 2285460 | 98.469 | 0.004 |
| 30 | 3475826 | 3476225 | 3475133 | 3475728 | 451.161 | 0.013 |
| 50 | 5749552 | 5749602 | 5749589 | 5749581 | 21.182 | 0.001 |
| 80 | 9024855 | 9025132 | 9025163 | 9025050 | 138.465 | 0.002 |
| 100 | 11133516 | 11142614 | 11138995 | 11138375 | 3740.027 | 0.034 |
| 120 | 12972895 | 13040253 | 12988312 | 13000487 | 28814.833 | 0.222 |

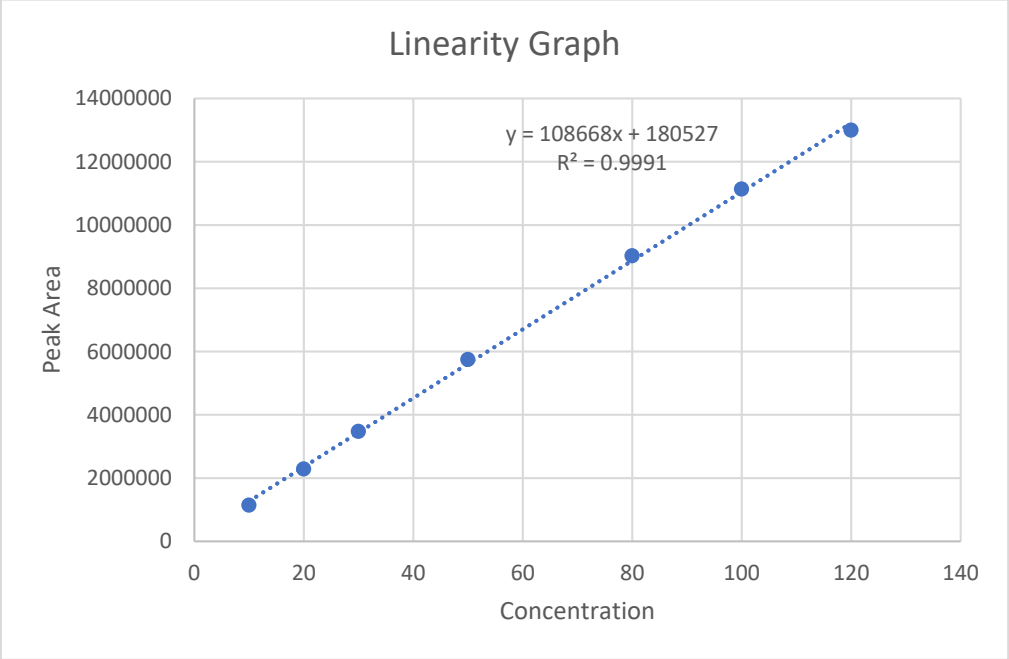



**Fig. 6.38. Linearity Graph for CIP (Method 4)**

Limit of Detection and Limit of Quantitation were calculated and found to be:

Limit of Detection, LOD (calculated) =4.266 µg/mL

Limit of Quantitation, LOQ (calculated) =14.221µg/mL

**Accuracy:**

Accuracy (% Recovery) was assessed in triplicate, at three different concentration levels of 80, 100 and 120% of the target concentration of active ingredient, by spiking a known amount of each of the standard to a sample of known concentration and then calculating the recovery and RSD (%) for each concentration as per procedure given in section 5.3.1.4.7 (pp 129).

**Table 6.79. Results of Accuracy of Method 4 for CIP**

| Level of addition (std) (%) | Amount of std added (µg/mL) | Avg. Peak Area | Conc (µg/mL) | Assay | Recovery | % recovery |
|---|---|---|---|---|---|---|
| 80 | 40 | 10126012 | 91.521 | 50.742 | 40.780 | 101.949 |
| 100 | 50 | 11140279 | 100.855 | 50.742 | 50.113 | 100.226 |
| 120 | 60 | 12095250 | 109.643 | 50.742 | 58.901 | 98.169 |

**Robustness**: The effect of changes in mobile phase proportion, pH and flow rate on retention time, peak area, number of theoretical plates and Tailing Factor was studied.

**Effect of mobile phase proportion:** Effect of mobile phase ratios 15:85 and 11:89 of methanol: buffer as compared to optimized ratio of 13:87 used in the method was studied.

**Table 6.80. Robustness: Effect of Mobile Phase Ratio**

| Drug/ Degradant | Mobile Phase Ratio | 15:85 | 13:87 (optimized) | 11:89 | % RSD |
|---|---|---|---|---|---|
| CIP | Avg. Peak Area (n=3) | 2014534 | 2004476 | 2007186 | 0.296 |
| | %RSD | 0.003 | 0.275 | 0.271 | |
| CDA1 | Avg. Peak Area (n=3) | 68492 | 71272 | 69477 | 1.776 |
| | %RSD | 0.488 | 0.089 | 0.620 | |
| CDA2 | Avg. Peak Area (n=3) | 90249 | 92695 | 91321 | 1.167 |
| | %RSD | 0.116 | 0.026 | 0.087 | |

**Effect of variation in pH**: pH of buffer was adjusted to 2.8 and 3.2 as compared to optimized pH of 3.0 used in the method (0.2 units variation)

**Table 6.81. Robustness: Effect of pH**

| Drug/ Degradant | pH | 2.8 | 3.0 (optimized) | 3.2 | % RSD |
|---|---|---|---|---|---|
| CIP | Avg. Peak Area (n=3) | 2058865 | 2004476 | 2067666 | 1.515 |
| | %RSD | 0.798 | 0.275 | 0.225 | |
| CDA1 | Avg. Peak Area (n=3) | 72513 | 71272 | 72737 | 1.111 |
| | %RSD | 0.176 | 0.089 | 1.078 | |
| CDA2 | Avg. Peak Area (n=3) | 91499 | 92695 | 90682 | 0.992 |
| | %RSD | 0.390 | 0.026 | 0.317 | |

**Effect of variation in mobile phase flow rate**: Flow rate of mobile phase was changed to 1.3 mL/min and 1.7 mL/min as compared to optimized flow rate of 1.5 mL/min used in the method (0.2 mL variation).

**Table 6.82. Robustness: Effect of Mobile Phase Flow Rate**

| Drug/ Degradant | Mobile Phase Flow Rate | 1.3 mL/min | 1.5 mL/min (optimized) | 1.7 mL/min | % RSD |
|---|---|---|---|---|---|
| CIP | Avg. Peak Area (n=3) | 2012867 | 2004476 | 2013301 | 0.258 |
| | %RSD | 0.078 | 0.275 | 0.045 | |
| CDA1 | Avg. Peak Area (n=3) | 72714 | 71272 | 72550 | 0.119 |
| | %RSD | 0.544 | 0.089 | 0.983 | |
| CDA2 | Avg. Peak Area (n=3) | 92714 | 92695 | 92785 | 0.451 |
| | %RSD | 0.667 | 0.026 | 0.600 | |

**Assay of Marketed Formulation:**

The proposed HPLC method was extended for the determination of CIP in tablets. The tablet used was Ciplox- 500 (batch no. SB60176) manufactured by Cipla Ltd. It was analysed using procedure given in section 5.3.1.4.9 (pp 131)**,** using proposed Method 4. Sample concentration of 50 µg/mL was injected into the chromatograph and resulting peak area from chromatogram recorded.

Average area (6 injections) for assay solution was calculated to be 5694577.

The concentration of sample OFL sample was calculated to be 50.742 µg/mL and assay results found to be complying 100.241 % with label claim (I.P. limits 90-110%).

The system suitability and validation results for Method 4 are summarised in Table 6.83.

**Table 6.83. System Suitability and Validation parameters using proposed Method 4 for CIP and degradants**

| System Suitability Parameters | CDA1 (Degradant 1) | CDA2 (Degradant 2) | CIP |
|---|---|---|---|
| N | 5713 | 7744 | 2497 |
| Tailing factor (Pk Sym) | 1.469 | 1.109 | 1.114 |
| %RSD of area(%RSD < 2%) | 0.088 | 0.026 | 0.001 |
| $R_t$ (min) | 5.125 | 8.900 | 9.977 |
| %RSD of $R_t$(%RSD < 2%) | 0 | 0.009 | 0.036 |
| $R_s$ with adjacent peak | 11.115 | 2.063 | NA |
| Linearity | NA | NA | y = 108668x +180527<br>$R^2$ = 0.9991 |
| LOD(µg/mL) | NA | NA | 4.266 |
| LOQ(µg/mL) | NA | NA | 14.221 |
| Precision (%RSD < 2%) | Complies | Complies | Complies |
| Assay of marketed formulation<br>(Limits 90-110%) | NA | NA | 100.241% |
| Accuracy | NA | NA | 98-102% |
| Robustness (%RSD < 2%) | Complies | Complies | Complies |

### 6.3 LCMS Studies

Instrument used: 6400 Series Triple Quadrupole B.08.00 (B8023.5 SP1) of Agilent Technologies (Courtesy: K.K. Birla Goa Campus of BITS Pilani)

### 6.3.1 Application of Method 3 for separation and subsequent detection of degradant via LCMS study

The stress degraded samples of LEV were prepared as stated under Table 5.4 (pp 136). The degraded samples (resulting from forced degradation of solutions of 20 µg/mL concentration) were subjected to LCMS studies for characterization of the components (parent drug and degradants) that have emerged as peaks in the HPLC, using Method 3.
Samples analysed: (1) LDA (Degradant produced by acid hydrolysis of LEV) and
(2) LDO (Degradant produced by oxidative degradation of LEV)
Method was developed, optimized and validated on HPLC in college laboratory. The validated method was then used for separation and detection of separated components on LC-MS at BITS Goa. Fragmentation was done with ESI, voltage calibrated to 135V for deriving optimum Collision Energy and spectrum run for mass range of 70 to 500.

#### 6.3.1.1. Mass Spectrum of acid hydrolysis degradant (LDA)

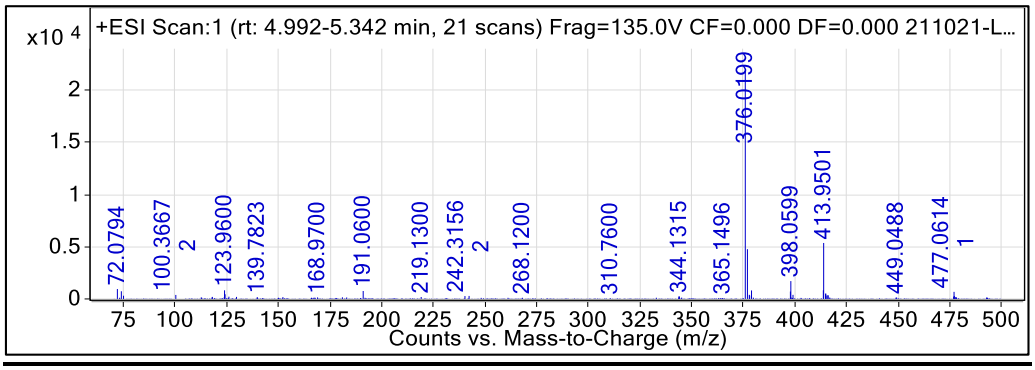



**Fig. 6.39. Mass Spectrum of acid hydrolysis degradant LDA**

### 6.3.1.2. Mass Spectra of oxidative degradant (LDO)

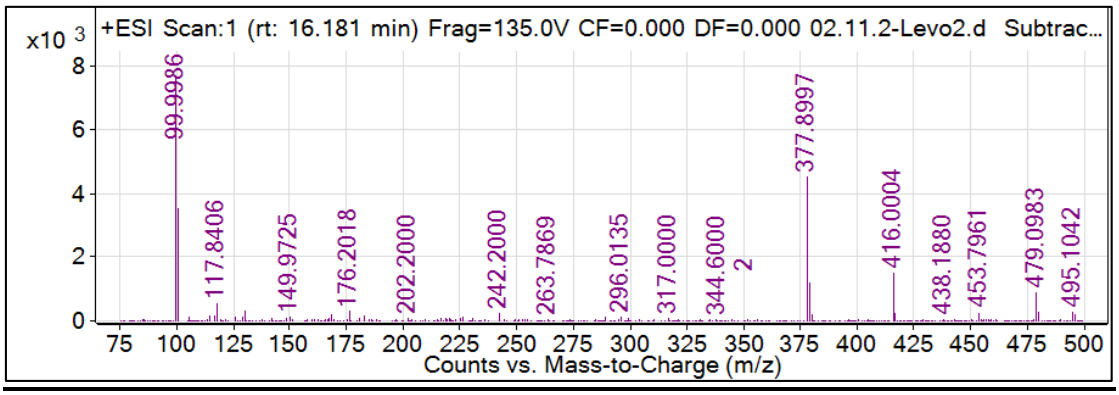



**Fig. 6.40. Mass Spectrum of oxidative degradant LDO**

In case of LDA as well as LDO, abundancy was observed for peak at m/z 377. The degradant that gives a peak in the mass spectrum with m/z 377 (M+16) is identified in literature as an N-oxide of LEV and confirmed with literature cited reports [41].

It was seen through a detailed search of literature that Levofloxacin-N-oxide is a major degradant in acid hydrolysis as well as oxidative degradation of LEV.

### 6.3.2 Application of Method 4 for separation and subsequent detection of degradant via LCMS study

The stress degraded samples of CIP were prepared as stated under Table 5.4 (pp 136). The degraded samples (resulting from forced degradation of solutions of 20 µg/mL concentration) were subjected to LCMS studies for characterization of components (parent drug and degradants) that have emerged as peaks in the HPLC, using Method 4.

Samples analysed: (1) CDA1 and CDA2 (Degradants produced by acid hydrolysis of CIP)

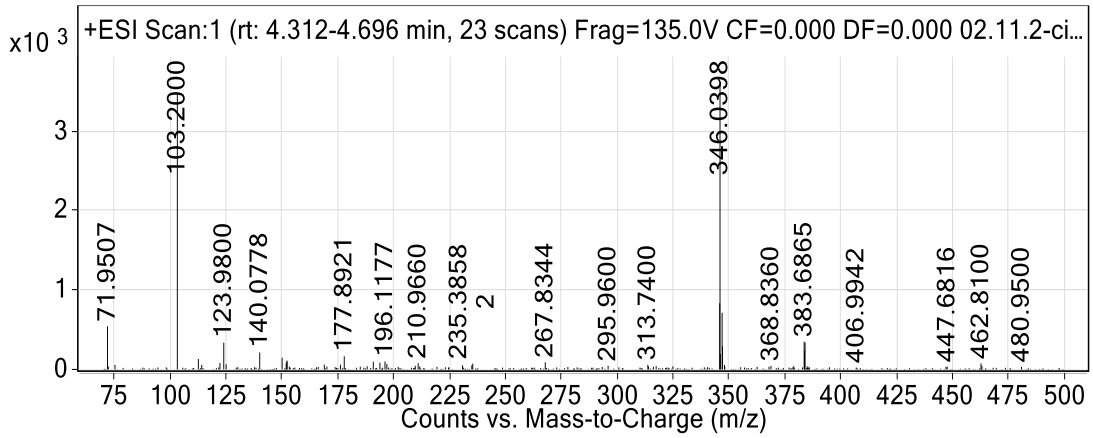



**Fig. 6.41. Mass Spectrum of oxidative degradant CDA1 of CIP**

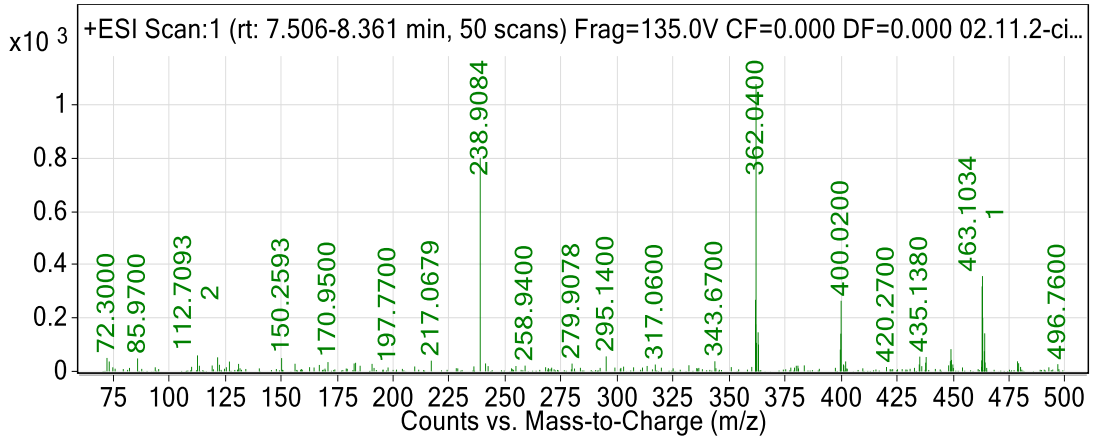



**Fig. 6.42. Mass Spectrum of oxidative degradant CDA2 of CIP**

LCMS studies have confirmed formation of degradants. Degradants that were predictable from Levofloxacin could be observed and confirmed.

It was observed that proposed validated stability indicating HPLC methods were compatible to LCMS. Some fragments produced in the mass spectrum could be correlated from published literature and are predictable for FQs.

### 6.4 Stability of Drugs under Forced Degradation

LEV and CIP were subjected to stress degradation under conditions that produce degradation.

Solutions were prepared as mentioned in section 5.3.1.1 (pp 122) and subjected to stress degradation studies as per procedures given in section 5.3.1.5 (pp 132).

The results are outlined in Table 6.84

**Table 6.84. Forced Degradation Studies**

| Stress | Drug | Stressing Agent | Strength of Acid/ Base/ Peroxide | Temperature | Duration | Observations |
|---|---|---|---|---|---|---|
| Acid Hydrolysis | CIP, LEV | HCL | 0.1 N, 1 N, 5 N | 65 °C | 6 h | Degradation observed in the form of additional peak |
| Base Hydrolysis | CIP, LEV | NaOH | 0.1 N, 1 N, 5 N | 65 °C | 6 h | Additional peak not observed |
| Oxidation | CIP, LEV | $H_2O_2$ | 30 % | 65 °C | 6 h | Degradation observed in the form of additional peak |
| Thermal | CIP, LEV | Dry Heat | ____ | 65 °C, 100 °C | 6 h | Additional peak not observed |
| Photolytic | CIP, LEV | Daylight | ____ | RT | 7 days | Additional peak not observed |

### 6.5 Stability of Drugs during Testing Conditions

The stability of the FQs under test conditions and parameters used in proposed methods was investigated. Solutions were prepared and subjected to stability testing as stated in section 5.3.1.6 (pp 135).

The results are presented in Table 6.85 and 6.86 for CIP, 6.87 and 6.88 for LEV, 6.89 and 6.90 for MOX, 6.91 and 6.92 for NOR, 6.93 and 6.94 for OFL.

#### 6.5.1 Stability Studies for CIP

**Table 6.85. CIP samples not protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 285223 | 289783 | 286132 | 287046 | 21.13546 | |
| 1 day | 284904 | 284293 | 285111 | 284769.3 | 20.45415 | 96.776 |
| 7days | 281212 | 281342 | 281297 | 281283.7 | 19.98345 | 94.549 |

**Table 6.86. CIP samples protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 285223 | 289783 | 286132 | 287046 | 21.13546 | |
| 1 day | 283445 | 284132 | 283899 | 283825.3 | 20.77013 | 98.271 |
| 7days | 281448 | 281515 | 281994 | 281652.3 | 20.08076 | 95.010 |

#### 6.5.2 Stability Studies for LEV

**Table 6.87. LEV samples not protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 1286703 | 1286951 | 1286856 | 1286837 | 20.07825 | |
| 1 day | 1286534 | 1286662 | 1286722 | 1286639 | 20.07602 | 99.989 |
| 7days | 1251571 | 1252235 | 1251438 | 1251748 | 19.07632 | 95.010 |

**Table 6.88. LEV samples protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 1286703 | 1286951 | 1286856 | 1286837 | 20.07825 | |
| 1 day | 1285698 | 1285462 | 1287923 | 1286361 | 20.07115 | 99.965 |
| 7days | 1252374 | 1253135 | 1250236 | 1251915 | 19.88738 | 99.049 |

### 6.5.3 Stability Studies for MOX

**Table 6.89. MOX samples not protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 1384783 | 1376755 | 1389280 | 1383606 | 19.97450 | |
| 1 day | 1382236 | 1385166 | 1383232 | 1383545 | 19.90123 | 99.633 |
| 7days | 1381335 | 1381236 | 1381070 | 1381214 | 19.72473 | 98.750 |

**Table 6.90. MOX samples protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 1384783 | 1376755 | 1389280 | 1383606 | 19.97450 | |
| 1 day | 1381335 | 1379321 | 1384663 | 1381773 | 19.87324 | 99.49308 |
| 7days | 1381436 | 1385643 | 1387765 | 1384948 | 19.78390 | 99.04577 |

### 6.5.4 Stability Studies for NOR

**Table 6.91.NOR samples not protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 548793 | 549391 | 549712 | 549298.7 | 20.96499 | |
| 1 day | 546675 | 546513 | 547525 | 546904.3 | 20.55441 | 98.042 |
| 7days | 545668 | 546245 | 545543 | 545818.7 | 19.19506 | 93.387 |

**Table 6.92. NOR samples protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 548793 | 549391 | 549712 | 549298.7 | 20.96499 | |
| 1 day | 546233 | 546397 | 546542 | 546390.7 | 20.57838 | 98.156 |
| 7days | 545878 | 546103 | 545946 | 545975.7 | 19.61749 | 95.331 |

### 6.5.5 Stability Studies for OFL

**Table 6.93.OFL samples not protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 1275889 | 1273243 | 1275680 | 1274937 | 20.06702 | |
| 1 day | 1274067 | 1271785 | 1281986 | 1275946 | 19.89278 | 99.131 |
| 7days | 1251571 | 1252235 | 1251438 | 1251748 | 19.07632 | 95.060 |

**Table 6.94.OFL samples protected from light**

| Time | Peak Areas | | | | Conc (µg/mL) | % drug remaining |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Avg | | |
| 0 | 1275889 | 1273243 | 1275680 | 1274937 | 20.067 | |
| 1 day | 1274339 | 1274768 | 1274189 | 1274432 | 19.890 | 99.118 |
| 7days | 1252374 | 1253135 | 1250236 | 1251915 | 19.379 | 96.570 |

From the results it is observed that the FQs show negligible degradation under conditions used for testing. The test conditions are soft on the molecules and do not encourage degradation.

### 6.6 Comparison of Proposed Methods with Existing methods

The proposed methods were compared with published methods for FQs as given under Review of Literature. As evident from the data obtained through literature survey, most HPLC methods reported for selected FQs of research interest involved sophisticated techniques of detection (fluorometric or MS), use of special columns (columns like phenylsilyl and chiral based columns which are beyond routine C18 and C8 type) and development through gradient elution. Methods that did not involve these techniques, were seen to contain higher proportion of organic component in the mobile phase or used a multicomponent mobile phase.

The proposed methods are isocratic and use UV detector / PDA detector, considered universal, column of C18 type with low proportion of organic phase was low.

Results of new developed methods on comparison to that of published methods confirms application to FQs of research interest and degradants with minor modifications. Methods with non-volatile buffers could be extendable to MS detection for identification of degradants.

In summary the methods developed were specific and also the FQs were found to remain stable during the analysis to provide true picture of the quality. The operational/ experimental variables did not elicit formation of degradants during the period of analysis.

Also, data from published methods can be utilized to generate Multiple Linear Regression models and these models can be used to predict retention times of other FQs and their related substances as reported in next section.

## 6.7 Prediction of Retention times.

### 6.7.1 Collection of Data

Published HPLC methods for estimation of multiple fluoroquinolones were selected (Table 3.14, pp 98) and attempts were made to establish relation between solute descriptors and retention time. Initially the Multiple Linear Regression analysis was applied to each selected method separately to determine the most relevant solute descriptors.

### 6.7.2 Calculation of Physicochemical Properties of Fluoroquinolones

Physicochemical properties of fluoroquinolones were calculated using OSIRIS DataWarrior and Chemaxon Software and are reported in Table 6.95.

**Table 6.95. Physicochemical properties of fluoroquinolones calculated using OSIRIS DataWarrior and Chemaxon Software**

| Name | cLogP | LogS | LogD | Topological Surface Area | Relative PSA |
|---|---|---|---|---|---|
| Ofloxacin | -0.34 | -2.74 | -1.56 | 252.39 | 0.24 |
| Ciprofloxacin | -1.53 | -3.32 | -2.03 | 232.79 | 0.25 |
| Norfloxacin | -1.65 | -2.86 | -2.08 | 232.29 | 0.25 |
| Lomefloxacin | -1.22 | -3.55 | -1.52 | 249.64 | 0.23 |
| Nalidixic acid | 0.54 | -2.67 | -1.00 | 173.66 | 0.31 |
| Levofloxacin | -0.34 | -2.736 | -2.15 | 252.39 | 0.24 |
| Moxifloxacin | -0.95 | -4.232 | -1.76 | 280.31 | 0.24 |
| Sparfloxacin | -1.45 | -4.466 | -1.27 | 269.66 | 0.27 |
| Gatifloxacin | -1.27 | -3.716 | -1.88 | 266.05 | 0.26 |
| Trovafloxacin | -0.55 | -6.027 | -7.95 | 271.03 | 0.27 |
| Cinoxacin | 0.96 | -2.661 | -6.44 | 181.16 | 0.41 |
| Pefloxacin | -0.26 | -2.494 | -1.57 | 243.39 | 0.20 |
| Enoxacin | -2.12 | -3.111 | -9.52 | 231.05 | 0.30 |

### 6.7.3 Application of Multiple Linear Regression Analysis

The models or regression equations were generated and evaluated for their prediction capability using F-test and $R^2$.

One of the selected HPLC methods (named as Method 10) which was further investigated by establishing multiple linear regression models has been described-

Description of Method 10:

Column: C18 (25cm x 4.6 mm, 5µ)

ACN (43): Aqueous (57; 10mM sodium dodecyl sulphate, 10mM TBAA. 25mM citric acid)

pH: 3.5; Flow: 1.0mL/min

Correlation Study between Molecular Descriptors and Retention Time is presented in Table 6.96.

**Table 6.96. Multiple Linear Regression Models obtained for Method 10 and the corresponding F, p and $R^2$ values**

| Sr. No. | Molecular Descriptor | Regression Equation | F-statistic | F from table | p-value | $R^2$ |
|---|---|---|---|---|---|---|
| 1 | cLogP | Rt = 5.37 - 0.97 cLogP | 1.47 | 7.71 | 0.2900 | 0.27 |
| 2* | LogS | Rt = 1.19 - 1.26 LogS | 40.70 | 7.71 | 0.0031 | 0.91 |
| 3 | TSA | Rt = -3.14 + 0.037 TSA | 7.60 | 7.71 | 0.0510 | 0.66 |
| 4 | Rel PSA | Rt = 9.66 - 13.36 Rel PSA | 1.60 | 7.71 | 0.2700 | 0.29 |
| 5 | LogD (pH3.5) | Rt = 7.42 + 0.61 LogD | 4.97 | 7.71 | 0.0900 | 0.55 |
| 6* | cLogP, LogS | Rt = 1.27 - 0.50 cLogP - 1.16 LogS | 60.41 | 9.55 | 0.0030 | 0.98 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 7 | cLogP, TSA | Rt = -3.65 + 0.14 cLogP + 0.039 TSA | 2.89 | 9.55 | 0.2000 | 0.66 |
| 8 | Rel PSA, cLogP | Rt = 8.03 - 0.43 cLogP - 8.44 Rel PSA | 0.64 | 9.55 | 0.5900 | 0.30 |
| 9* | LogS, TSA | Rt = -1.64 - 0.97 LogS + 0.016 TSA | 93.50 | 9.55 | 0.0020 | 0.98 |
| 10* | LogS, Rel PSA | Rt = 3.41 - 1.16 LogS - 6.55 Rel PSA | 52.86 | 9.55 | 0.0050 | 0.97 |
| 11 | TSA, Rel PSA | Rt = -13.68 + 15.86 Rel PSA + 0.062 TSA | 4.71 | 9.55 | 0.1200 | 0.76 |
| 12* | LogS, LogD | Rt = 2.55 - 1.05 LogS + 0.23 LogD | 36.51 | 9.55 | 0.0080 | 0.96 |
| 13 | LogD, cLogP | Rt = 7.78 + 0.25 cLogP + 0.69 LogD | 1.92 | 9.55 | 0.2900 | 0.56 |
| 14 | LogD, TSA | Rt = -2.74 + 0.035 TSA + 0.027 LogD | 2.85 | 9.55 | 0.2000 | 0.66 |
| 15 | LogD, Rel PSA | Rt = 0.40 + 34.51 Rel PSA + 1.66 LogD | 5.33 | 9.55 | 0.1000 | 0.78 |
| 16* | LogS, TSA, cLogP | Rt = -0.66 - 0.26 cLogP - 1.02 LogS + 0.011 TSA | 99.43 | 19.16 | 0.0100 | 0.99 |
| 17* | cLogP, LogS, Rel PSA | Rt = 2.27 - 0.31 cLogP - 1.15 LogS - 3.06 Rel PSA | 32.27 | 19.16 | 0.0300 | 0.98 |
| 18* | cLogP, LogS, LogD | Rt = 1.63 - 0.40 cLogP - 1.12 LogS + 0.063 LogD | 28.74 | 19.16 | 0.0300 | 0.98 |
| 19* | LogS, TSA, Rel PSA | Rt = -1.20 + 0.015 TSA - 0.99 LogS - 0.63 Rel PSA | 41.78 | 19.19 | 0.0200 | 0.98 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 20* | LogS, TSA, LogD | Rt = -2.26 + 0.018 TSA - 0.98 LogS - 0.042 LogD | 42.7 | 19.16 | 0.0200 | 0.98 |
| 21* | LogS, LogD, Rel PSA | Rt = 5.19 - 23.71 Rel PSA - 1.51 LogS - 0.67 LogD | 48.41 | 19.16 | 0.0200 | 0.99 |
| 22 | cLogP, LogD, TSA | Rt = -2.33 + 0.035 TSA + 0.21 cLogP + 0.10 LogD | 1.3 | 19.16 | 0.4600 | 0.66 |
| 23 | cLogP, LogD, Rel PSA | Rt = -3.44 + 47.31 Rel PSA - 0.88 cLogP + 1.76 LogD | 3.46 | 19.16 | 0.2300 | 0.84 |
| 24 | cLogP, TSA, Rel PSA | Rt = -18.39 + 0.065 TSA - 0.88 cLogP + 27.64 Rel PSA | 2.97 | 19.16 | 0.2600 | 0.82 |
| 25 | LogD, TSA, Rel PSA | Rt = -10.50 + 0.037 TSA + 1.08 LogD + 35.40 Rel PSA | 5.58 | 19.16 | 0.1600 | 0.89 |

The bigger the F-value calculated (larger than the tabulated F-value), more likely is the model/ regression equation to explain the dependency of the Y (Retention Time) on the X-variables (Molecular Descriptors). $R^2$ expresses the ratio of the variance that can be explained by the regression model. Higher $R^2$ values indicate majority of the variance can be explained.

### 6.7.4 Calculation of Physicochemical properties of FQ related substances

Using the criteria for evaluation, those models which showed promise, namely methods at Sr. No.s 2, 6, 9, 10, 12, 16, 17, 18, 19, 20 and 21 (marked with an *) in Table 6.96, were further investigated for their prediction capacity by using reported degradants / related substances of Levofloxacin.

Physicochemical properties of these degradants / related substances were also calculated using OSIRIS DataWarrior and Chemaxon Software.

**Table 6.97. Physicochemical Properties of degradants/ related substances calculated using OSIRIS DataWarrior and Chemaxon Software**

| Imp/ Deg No. | Name | cLogP | LogS | LogD | | | Topological Surface Area | Relative PSA |
|---|---|---|---|---|---|---|---|---|
| | | | | pH 2.5 | pH 3.4 | pH 3.5 | | |
| 1 | Levofloxacin | -0.34 | -2.74 | -1.86 | -1.52 | -1.45 | 252.39 | 0.24 |
| 2 | Desmeth-levofloxacin | -1.73 | -3.10 | -2.06 | -2.05 | -2.05 | 241.29 | 0.28 |
| 3 | D-Levofloxacin | -0.34 | -2.74 | -1.86 | -1.52 | -1.45 | 252.39 | 0.24 |
| 4 | Desmeth-D-Levofloxacin | -1.73 | -3.10 | -2.06 | -2.05 | -2.05 | 241.29 | 0.28 |
| 5 | Levofloxacin-N-oxide | -0.39 | -0.57 | 0.35 | 0.37 | 0.37 | 254.26 | 0.24 |
| 6 | Desfluoro-levofloxacin | -0.44 | -2.42 | -2.06 | -1.93 | -1.9 | 246.04 | 0.24 |
| 7 | Descarboxyl-Levofloxacin | 1.42 | -3.14 | -1.45 | -1.12 | -1.05 | 230.08 | 0.15 |
| 8 | Difluoro-levofloxacin | 0.91 | -3.45 | 1.75 | 1.74 | 1.74 | 184.34 | 0.29 |
| 9 | Desethylene-Levofloxacin | -1.51 | -2.98 | -2.54 | -2.52 | -2.51 | 240.69 | 0.31 |
| 10 | Levofloxacin-ethylester | 1.42 | -3.16 | -1.13 | -0.79 | -0.72 | 282.06 | 0.20 |

### 6.7.5 Prediction of Retention Times for Related Substances of LEV

The retention times of these related substances of LEV were predicted using the regression models generated and have been reported in Table 6.98.

**Table 6.98. Predicted Retention times for degradants/ related substances using regression models generated earlier for each method/ study**

| Imp / Deg No. | Name | Retention Times (in mins) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Model 1 | Model 2 | Model 3 | Model 4 | Model 5 | Model 6 | Model 7 | Model 8 | Model 9 | Model 10 | Model 11 |
| 1 | Levofloxacin | 4.64 | 4.62 | 5.05 | 5.03 | 5.09 | 5.00 | 4.80 | 4.74 | 5.15 | 5.03 | 4.67 |
| 2 | Desmeth-levofloxacin | 5.09 | 5.73 | 5.23 | 5.16 | 5.33 | 5.60 | 5.51 | 5.66 | 5.31 | 5.21 | 4.59 |
| 3 | D-Levofloxacin | 4.64 | 4.62 | 5.05 | 5.03 | 5.09 | 5.00 | 4.80 | 4.74 | 5.15 | 5.03 | 4.67 |
| 4 | Desmeth-D-levofloxacin | 5.09 | 5.73 | 5.23 | 5.16 | 5.33 | 5.60 | 5.51 | 5.66 | 5.31 | 5.21 | 4.59 |
| 5 | Levofloxacin-N-oxide | 1.90 | 2.12 | 2.98 | 2.48 | 3.23 | 2.82 | 2.30 | 2.44 | 3.02 | 2.86 | 0.04 |
| 6 | Desfluoro-levofloxacin | 4.24 | 4.30 | 4.65 | 4.63 | 4.66 | 4.63 | 4.45 | 4.40 | 4.74 | 4.62 | 4.35 |
| 7 | Descarboxyllevofloxacin | 5.14 | 4.20 | 5.08 | 6.09 | 5.60 | 4.70 | 4.99 | 4.51 | 5.26 | 5.00 | 7.16 |
| 8 | Difluoro-levofloxacin | 5.53 | 4.81 | 4.65 | 5.53 | 6.57 | 4.65 | 5.08 | 5.24 | 4.80 | 4.36 | 2.44 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | Desethylen elevofloxac in | 4.94 | 5.47 | 5.10 | 4.80 | 5.10 | 5.41 | 5.20 | 5.41 | 5.16 | 5.09 | 3.91 |
| 10 | levofloxaci n-ethylester | 5.18 | 4.23 | 5.94 | 5.76 | 5.71 | 5.30 | 4.85 | 4.56 | 6.04 | 5.95 | 5.68 |

Model 1: Rt = 1.19 - 1.26 LogS

Model 2: Rt = 1.27 - 0.50 cLogP - 1.16 LogS

Model 3: Rt = -1.64 - 0.97 LogS + 0.016 TSA

Model 4: Rt = 3.41 - 1.16 LogS - 6.55 Rel PSA

Model 5: Rt = 2.55 - 1.05 LogS + 0.23 LogD

Model 6: Rt = -0.66 - 0.26 cLogP - 1.02 LogS + 0.011 TSA

Model 7: Rt = 2.27 - 0.31 cLogP - 1.15 LogS - 3.06 Rel PSA

Model 8: Rt = 1.63 - 0.40 cLogP - 1.12 LogS + 0.063 LogD

Model 9: Rt = -1.20 + 0.015 TSA - 0.99 LogS - 0.63 Rel PSA

Model 10: Rt = -2.26 + 0.018 TSA - 0.98 LogS - 0.042 LogD

Model 11: Rt = 5.19 - 23.71 Rel PSA - 1.51 LogS - 0.67 LogD

### 6.7.6 Application of ANOVA to Predicted Retention Times

One way ANOVA was then used to analyse the results obtained and prove that they are not significantly different.

The statistical test used was One-Way ANOVA test, using F distribution df(10,99) (right tailed). The results are presented in Table 6.99.

**Table 6.99. One-Way ANOVA test for Predicted Retention Times**

| Source | DF | Sum of Square | Mean Square | F Statistic | P-value |
|---|---|---|---|---|---|
| **Groups** (between groups) | 10 | 6.4453 | 0.6445 | 0.6095 | 0.8025 |
| **Error** (within groups) | 99 | 104.6988 | 1.0576 | | |
| **Total** | 109 | 111.1441 | 1.0197 | | |

F- value from Table: 1.93 (Table 6.99)

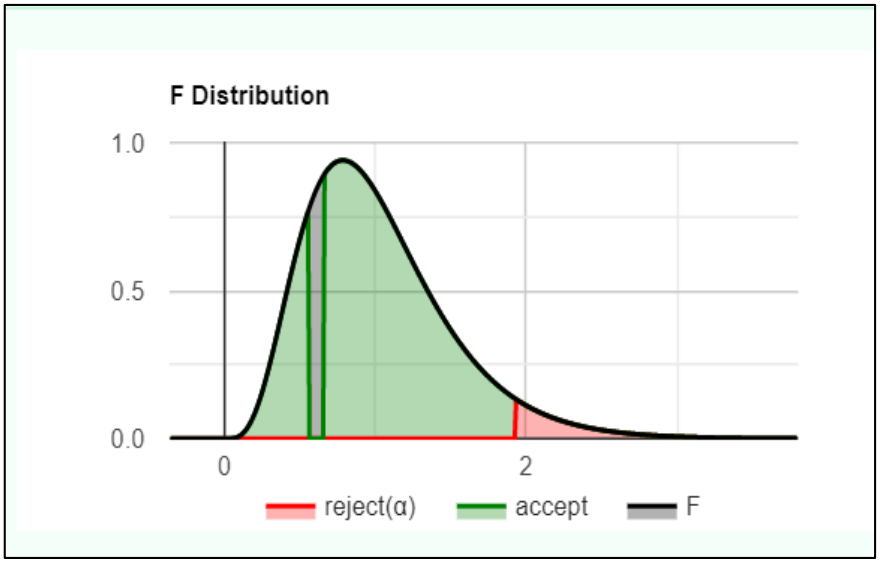



**Fig. 6.43. One-Way ANOVA test for Predicted Retention Times**

Since the calculated F-value (0.6095) is less than the tabulated F-value (1.93), it can be statistically concluded that the predicted retention times are not significantly different.

It has been observed that retention times of other fluoroquinolones and their related substances/ degradants can be predicted with high degree of confidence and accuracy provided the sample size of training set of fluoroquinolones is sufficiently large.

It was also found that studies/ methods which involved lower sample size (4 or less) were not suitable for formulation of regression or prediction models for predicting retention times.

Available data sets on drugs selected were found to be lesser and tabulation of the data could not provide an algorithm that could correlate retention profile with physicochemical attributes of the drug.

# *CHAPTER 7*

## *SUMMARY AND CONCLUSIONS*

## 7. Summary and Conclusions

Analytical techniques assure that the drug under investigation, or its formulated product, meets with the standards set for safety and efficacy. In order to do this reliably, the analytical method needs to be selective and specific so that the results are a true reflection of the quality of the sample. The method in itself should not employ conditions conducive to alteration in the drug molecule due to degradation during testing, thereby giving erroneous and misleading results. Hence it is of utmost importance that the stability profile of the drug candidate is studied and analytical methods developed in a way that avoids creating conditions encouraging drug instability during testing.

Testing methods being based on molecular properties linked to chemical structures, it is generally assumed that drug molecules bearing similar structural features will be analysed by same or similar methods. In the past on the basis of the chemistry of molecules similar process were used in volumetric analysis whereas in the current compendia diverse conditions (stationary phase, mobile phase) are recommended for use while performing assay.

It is envisaged here to identify certain parameters that favor use of stationary phase - mobile phase systems to such molecules that possess a common structural template. As a study case, Fluoroquinolones (FQs) were selected that offers several dozens of molecules bearing common structural features. The FQ class of compounds comprises of a wide range of drugs which have the same FQ nucleus but bearing widely differing functional groups, a fact that results in the drugs exhibiting diverse physical and chemical properties. While selecting drugs belonging to the class due consideration was given to their spectrum of activity and physicochemical properties.

The proposed newer methods were compared with published methods for FQs for the performance. As evident from the data obtained through literature survey, most HPLC methods reported for selected FQs of research interest involved sophisticated techniques of detection (fluorometric or MS), use of special columns and development through gradient elution. Methods that did not involve such techniques, were seen to contain higher proportion of organic component in the mobile phase or used gradient mobile phase.

The newer methods proposed are isocratic and use UV detector / PDA detector, considered universal, column of C18 type with low proportion of organic phase and were successfully applied to formulations, and may be applied to other FQs as single APIs.

Results of new developed methods on comparison to that of published methods confirms application to FQs of research interest and degradants with minor modifications. Response appears to be restricted to FQ core, additional diverse functional groups lead to modifications in the experimental conditions. Methods with volatile buffers could be extendable to MS detection for identification of degradants.

Yet another primary objective of predicting retention times of molecules using appropriate molecular descriptors and statistical regression models, was achieved by collating critical data like retention time, mobile phase systems, stationary phases and such other experimental attributes like temperature, detection wavelength from published methods to enable generation of Multiple Linear Regression models. It was demonstrated that these models could be successfully used to predict retention times of other FQs and their related substances. Factors like cLogP, LogD, LogS, Topological surface area,

Relative polar surface area were identified to be molecular descriptors that affect retention time.

The secondary objective involved study of stability profiles of selected fluoroquinolones under stressed conditions involving hydrolysis, temperature, light and oxidation that generally the drug gets exposed to during analysis. Assessment of stability profiles recorded ensured that proposed methods did not invoke such conditions that endanger the structural integrity of the FQ molecules.

In summary, the process in the methods developed were specific and ensured FQs remain stable during the period of analysis thus showcasing true picture of quality. The operational/ experimental variables did not elicit formation of degradants during the period of analysis.

## Future Scope

There have been instances wherein batches of drugs have been recalled due to reasons arising out of deficiencies in testing method wherein impurities have been generated. It is highly recommended that stability profiles of drugs be studied thoroughly with conditions generally adopted during analytical method development. Drugs containing hydrolysable groups like esters and amides, oxidizable groups like ketones require judicial application of experimental conditions. Molecules with such groups are expected to pose a challenge to the analyst. Such studies would ensure testing methods to be strong and could reflect eventually true quality of the sample.

Using molecular descriptors to develop MLR models and predict retention times, will help tremendously in analytical method development to shorten duration and lessen efforts put into development of methods, by predicting the time of elution of other related substances, including other members of the class or degradants and impurities. Preliminary steps and numerous trials can be skipped by taking help of available computing tools, making the process of method development simpler, easier and quicker.

# *CHAPTER 8*

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# *CHAPTER 9*

## *APPENDED DOCUMENTS*

## 9. List of Appended Documents

### List of Publications

9.1 Kudchadkar SS, N Pai SP. QbD Based RP-HPLC Method Development for Five Fluoroquinolone Anti-Bacterials-Through Creation of Design Space for Critical Attributes. Int J Pharm Sci Res 2019;10:4907.

9.2 Kudchadkar SS, Pai S. QbD Based Development of Two RP-HPLC Methods for Levofloxacin and Its Acid Degradation Product-Through Creation of Design Space for Critical Attributes and Application of ANOVA. Indian Journal of Natural Sciences 2022;13.

### List of Presentations

9.3 Paper titled "QbD Based Analytical Method Development through Creation of Design Space for Critical Attributes - Application on RP=HPLC Method Development for fivr Fluoroquinolone Antibacterials" presented at 70$^{th}$ IPC at New Delhi (2018).

9.4 Paper titled "Development of simple RP-HPLC Methods for Levofloxacin and its Degradation Products" at 6$^{th}$ Annual International Conference on IPR at Goa College of Pharmacy, Panaji, Goa (2021).

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# QbD BASED RP-HPLC METHOD DEVELOPMENT FOR FIVE FLUOROQUINOLONE ANTI-BACTERIALS - THROUGH CREATION OF DESIGN SPACE FOR CRITICAL ATTRIBUTES

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**Keywords:**

Fluoroquinolones, RP-HPLC, QbD, Design space

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**ABSTRACT:** Quality by Design (QbD) has become an important concept for the pharmaceutical industry, and ICH recommends this concept through the Guideline Q8 (R2) wherein if the method is proved to be rugged and robust within the limits of a Design Space then approval may be requested for such a Design Space, and the method does not need redevelopment, revalidation, and reapproval during transfers, thereby saving significant time and resources. In this study, the QbD approach was used to develop a reverse-phase–high-performance liquid chromatography (RP–HPLC) method that could be applied for the estimation of several antibacterial agents of the fluoroquinolone group. The method was applied to five selected fluoroquinolones, namely, ciprofloxacin, levofloxacin, norfloxacin, moxifloxacin, and ofloxacin. The method developed used C18 column of make Phenomenex (250 mm × 4.6 mm, 5 µm) with mobile phase comprising of methanol and phosphate buffer (pH 3.0) in the proportion 70:30 at a flow rate of 1.25 ml/min and detection was carried out using a PDA detector at 294 nm. The method while being validated as per ICH guidelines, using ANOVA, simultaneously created a design space which set limits for critical attributes affecting the robustness. Any variations in these parameters within the design space does not affect method performance, making the method easily transferrable.

**INTRODUCTION:** The fluoroquinolones (FQs) are a family of broad-spectrum, systemic antibacterial agents that are being used widely for the treatment of respiratory and urinary tract infections. The first quinolone was reported in the early 1960s, with the isolation of 7-chloro-l-ethyl-1, 4-dihydro-4-oxyquinoline-3-carboxylic acid, a by-product of the commercial preparation of chloroquine.

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Since this particular substance was discovered to have anti-bacterial activity, it was used to produce nalidixic acid, which is a 1, 8-naphthyridine [1]. After the passage of many decades, the members of this class, even the older agents are still finding a use for various treatments [2, 3]. The concept of Quality by Design has gained a lot of importance in Pharma Industry, and any analytical method developed using QbD principles does not need redevelopment, revalidation, and reapproval during transfers, provided one work within the limits of the approved Design Space, thereby saving time and resources [4].

The work reported here describes our efforts towards using QbD principles and creation of a Design Space as recommended by ICH Q8 (R2) in

the development and validation of a common analytical method for five selected FQs, namely, ciprofloxacin (CIP), levofloxacin (LEV), moxifloxacin (MOX), norfloxacin (NOR) and ofloxacin (OFL), the chemical structures of which have been shown in **Fig. 1**. A literature survey did not reveal any common method for these five FQs. Most of the reported analytical methods aimed at simultaneous estimation of FQs making the method complicated, involving gradient elution and fluorescence or other highly sophisticated detection and quantitation methods [5, 6, 7, 8, 9]. As such, even though one often deals with multiple members of this class, they are not used in combination with each other. Hence, it was thought to develop an analytical method which was simple and quick and could be commonly used for the majority of the class. The method has been developed and validated as per ICH guidelines[10] and using QbD approach [11, 12, 13, 14].

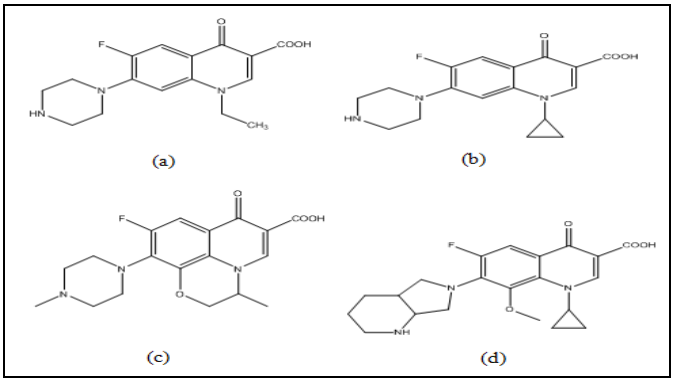


**FIG. 1: STRUCTURES OF (A) NOR, (B) CIP, (C) OFL AND LEV* AND (D) MOX.** *LEV is levorotatory whereas OFL is a racemic mixture, having the same chemical structure

## MATERIALS AND METHODS:

**Chemicals:** The chemicals used were methanol (HPLC grade of Merck), HPLC grade water obtained in-house from BioAge Ultra Water Purification system, orthophosphoric acid and potassium dihydrogen orthophosphate (AR grade)

The pure drugs were obtained as gift samples from Abaris Healthcare Pvt. Ltd., Ahmedabad, Gujarat, India.

The experimental work was performed at the Central Instrument Laboratory of Goa College of Pharmacy, Panaji, Goa, India.

**Equipment:** The following equipment/instruments were used: Electronic Weighing Balance (Wensar), sonicator (Citizen), pH meter (Labtronics), HPLC (Jasco) with ChromNav software and Phenomenex C18 column.

**Chromatographic Conditions:** The HPLC system used was Jasco CO-4061 with Autosampler (AS-4050) and a PDA detector and built-in degasser. The system used ChromNav software.

After various trials, Phenomenex C18 column (250 mm × 4.6 mm i.d., 5µ) was selected with a mobile phase composed of methanol and phosphate buffer.

The mobile phase used was 70 parts methanol and 30 parts buffer. Phosphate buffer pH 3.0 was prepared by dissolving Potassium dihydrogen orthophosphate in water and adjusting the pH to 3.0 by using orthophosphoric acid. Mobile phase components were sonicated for 15min and filtered through 0.45µ membrane filters before use.

The flow rate was maintained at 1.25 ml/min and a detector set to wavelength 294 nm. Autosampler used had variable loop volume 0-100 µl, and in this method, 20 µl was injected. The system had a column oven making it possible to program column temperatures during the run. After a trial at various temperatures, it was decided to set the column temperature at 40 °C throughout the method.

Optimized chromatographic conditions for selected fluoroquinolones are shown in **Table 1**.

**TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS**

| Parameters | Values |
|---|---|
| Stationary phase (column) | C18 (250 mm × 4.6 mm, 5 µm) |
| Mobile phase | Methanol: phosphate buffer pH 3.0 (70:30, v/v) |
| Flow rate (mL/min) | 1.25 |
| Run time (min) | 5.0 |
| Column Temperature | 40°C |
| Injection Volume (µl) | 20 |
| Detection Wavelength (nm) | 294 |

**Method Development:**

**Selection and Preparation of Mobile Phase:** Mobile phases containing methanol, water, acetonitrile, and buffers at different pH were tried in different proportions and at different flow rates. Satisfactory peaks were obtained at a flow rate of 1.25ml/min with a mobile phase made up of 70 parts methanol and 30 parts of phosphate buffer

pH3.0. The 20 mM buffer was prepared by dissolving 2.72 gm of potassium dihydrogen orthophosphate per liter of buffer solution and adjusting pH to 3.0 by using orthophosphoric acid with the help of pH meter. Both components of the mobile phase were filtered through 0.45µm membrane filters by application of vacuum and sonicated for 15 min before introducing into the system.

**Preparation of Standard Stock Solutions:** The standard solutions of the drugs were prepared in methanol. A quantity of 25 mg of each drug was weighed and dissolved in methanol in 25 ml volumetric flasks, to give standard stock solutions of 1000 µg/mL of each drug. The standard stock solutions were further diluted with methanol to obtain required concentrations of each drug. All solutions, including stock solution, were freshly prepared each day.

**Preparation of Calibration Curve:** Volumes of standard stock solutions of each drug were transferred to a 10 mL volumetric flask and diluted up to the mark with methanol. Aliquots were taken in such a way to obtain final concentrations in the range of 10-60 µg/mL for each drug.

Three injections of 20 µl of each concentration were analyzed using optimized conditions. Each reading or peak area recorded was average or meant of three readings.

Calibration curves were plotted for each drug by plotting peak areas recorded for each concentration on the y-axis and the concentration of the drug on the x-axis. The coefficient of determination ($R^2$) was calculated for the calibration curve of each drug.

**Method Validation:** The method developed was validated as per ICH guidelines by evaluating parameters such as accuracy, precision, linearity, robustness, ruggedness, detection, and quantification limits. The results were evaluated considering acceptable limits as less than 2% for Relative Standard Deviation (RSD).

**Precision:** The precision of the developed method was confirmed for each of the drugs. The peak areas recorded by actual analysis of six replicate injections of a standard concentration of each drug. The precision of the method was also checked in terms of the intra- and inter-day variation in the peak areas by calculating the RSD.

**Accuracy:** The accuracy of the method was tested for each of the drugs by spiking a known concentration of each drug at three different concentration levels, namely 80%, 100%, and 120%, and then comparing the difference between the expected/theoretical value and the concentration determined by the method.

**Linearity:** A stock solution of 1000 µg/mL in methanol was prepared for each drug, namely, CIP, LEV, MOX, NOR, and OFL. From this stock, working standard solutions were prepared for each of the drugs, in the range of 10 to 60 µg/mL and injected into the HPLC system. It was proved that each drug shows linearity in the range of 10–60 µg/mL. The calibration graph (obtained by plotting peak areas of the drug under consideration versus its concentration) was generated by replicate analysis at all concentration levels, and the linearity of the relationship was established using Microsoft Excel® program.

**Robustness and QbD Approach to Designate Critical Attributes:** Robustness of the developed method for selected fluoroquinolones was confirmed by checking the effect of variation in critical parameters, namely, flow rate, pH and proportion of mobile phase components. ANOVA was applied to statistically prove robustness.

**Detection and Quantification Limit:** The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the linearity curve plotted. The formulae used for computing these values were:

$$LOD = 3.3\sigma/s$$

$$LOQ = 10\sigma/s$$

Where σ is the standard deviation of the y-intercept of the regression line, and s is the slope of the calibration curve.

## RESULTS AND DISCUSSION:

### Method Development:

**Chromatographic Separation:** After a number of trials, chromatographic conditions were optimized and selected based on System Suitability parameters.

The optimized chromatographic conditions are reported in **Table 1**.

Representative HPLC Chromatograms are shown in **Fig. 2**.

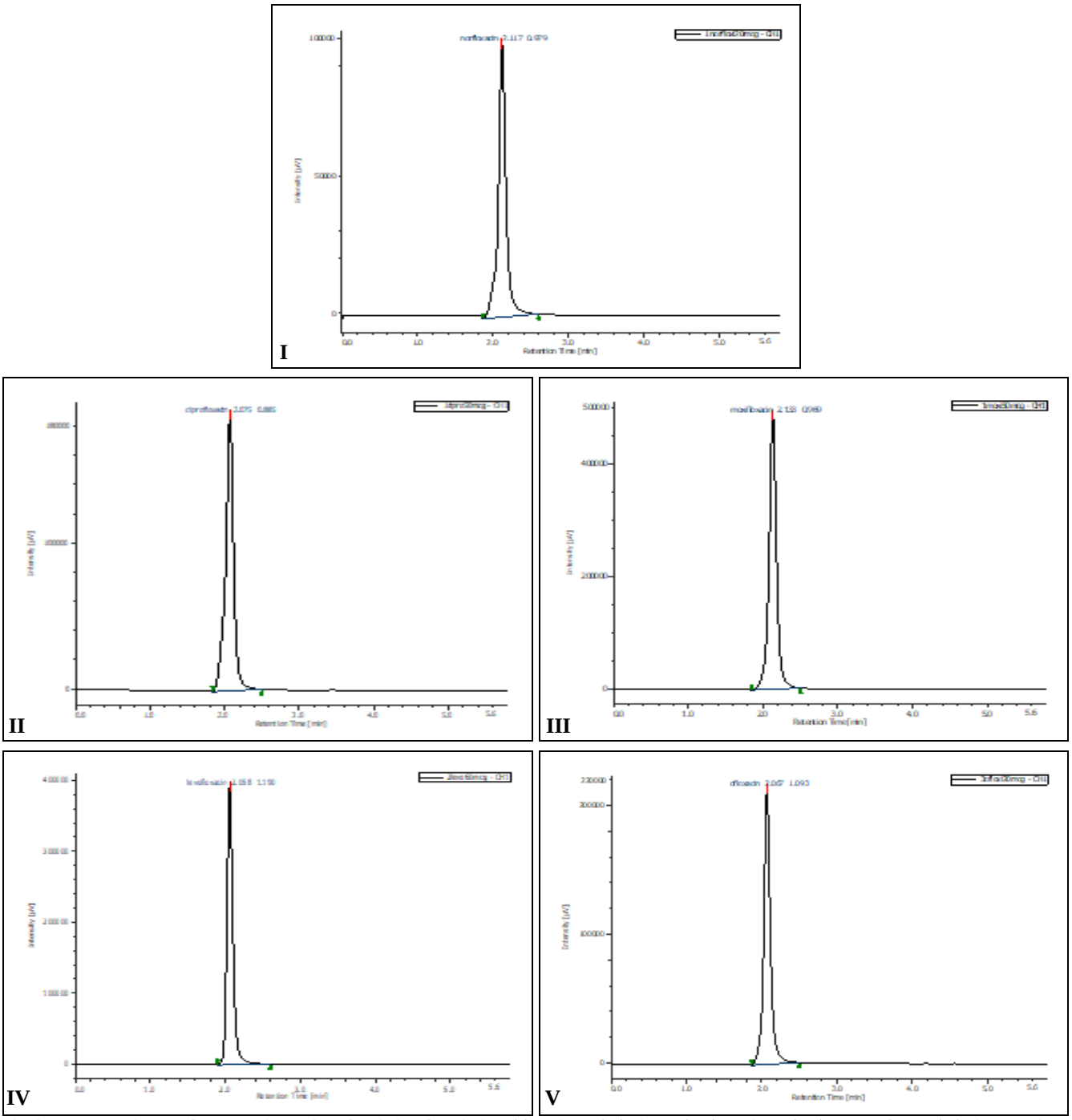


**FIG. 2: REPRESENTATIVE CHROMATOGRAMS OF (I) NOR, (II) CIP, (III) MOX, (IV) LEV AND (V) OFL USING C18 COLUMN WITH MOBILE PHASE OF METHANOL AND PHOSPHATE BUFFER, pH 3.0 (70:30) AT 1.25 mL/min AND DETECTION AT 294 nm**

System Suitability parameters for each drug were checked and are tabulated in **Table 2**.

**TABLE 2: SYSTEM SUITABILITY PARAMETERS**

| S. no. | Parameters | Acceptance Criteria | CIP | LEV | MOX | NOR | OFL |
|---|---|---|---|---|---|---|---|
| 1 | Theoretical Plates | >1000 | 2327 | 3793 | 2380 | 2848 | 4059 |
| 2 | Tailing factor | <2 | 0.891 | 0.890 | 0.877 | 0.960 | 0.877 |
| 3 | RSD of area | <2% | 0.196 | 0.390 | 0.051 | 0.075 | 0.067 |
| 4 | RSD of Ret.Time | <1% | 0.197 | 0.197 | 0.280 | 0.017 | 0.156 |

**Calibration Curve:** The correlation coefficients ($R^2$) for each of the three drugs under consideration and also the linearity equations are displayed in **Table 3**.

**TABLE 3: CORRELATION COEFFICIENTS AND LINEARITY EQUATIONS**

| S. no. | Drug | $R^2$ | Linearity equation |
|---|---|---|---|
| 1 | CIP | 0.999 | y = 852.1x + 2198 |
| 2 | LEV | 0.999 | y = 69819x + 134352 |
| 3 | MOX | 0.999 | y = 66363x + 97550 |
| 4 | NOR | 0.999 | y = 36901x + 1747.1 |
| 5 | OFL | 0.999 | y = 62185x + 10414 |

**Method Validation:** The method was validated and applied to marketed formulations. The results are reported in **Table 4**.

**TABLE 4: VALIDATION RESULTS**

| | CIP | LEV | MOX | NOR | OFL |
|---|---|---|---|---|---|
| Linearity range(µg/ml) | 10-60 | 10-60 | 10-60 | 10-60 | 10-60 |
| LOD(µg/ml) | 1.79 | 1.57 | 1.57 | 0.799 | 1.39 |
| LOQ(µg/ml) | 5.96 | 5.24 | 5.22 | 2.667 | 4.64 |
| Precision | Complies | Complies | Complies | Complies | Complies |
| Assay (Limits 90-110%) * | 100.05% | 104.19% | 103.17% | 106.75% | 98.004% |
| Accuracy | 101-102% | 95-101% | 96-102% | 102-105% | 96-105% |
| Robustness** | Complies | Complies | Complies | Complies | Complies |

*Performed on marketed formulations; **Proved through the application of ANOVA

**TABLE 5: CRITICAL ATTRIBUTES AND RANGES ANALYSED**

| S. no. | Critical Attribute | Value | Range |
|---|---|---|---|
| 1 | % of Methanol | 70% | 68-72% |
| 2 | pH of Buffer | 3.0 | 2.8-3.2 |
| 3 | Flow Rate | 1.25ml/min | 1.05-1.45ml/min |

**Creation of Design Space for Critical Attributes through QbD:**

**Identification of Critical Attributes and their Range:** The critical attributes in designing a robust and easily transferrable method were identified and tested over ranges, as shown in **Table 5**.

**Application of ANOVA to Creation of Design Space:** Application of statistical analysis in the form of ANOVA proved the method to be robust and helped create a Design Space for each critical parameter as enlisted in **Table 6**.

The *f*- ratio shows that there is no significant difference in results of the method if one works within the Design Space studied, thus displaying that method need not be redeveloped and revalidated during transfers.

**TABLE 6: APPLICATION OF ANOVA TO PROVE ROBUSTNESS OF EACH CRITICAL ATTRIBUTE (CA)**

| Drug | CA | n | ΣX | Mean | $\Sigma X^2$ | SD | *f*-ratio | *p*-value |
|---|---|---|---|---|---|---|---|---|
| CIP | % of Methanol | 9 | 23.71 | 2.63 | 62.44 | 0.0033 | 1.00 | 0.420 |
| | pH of Buffer | 9 | 23.58 | 2.62 | 61.75 | 0.0053 | 7.00 | 0.027 |
| | Flow Rate | 9 | 23.68 | 2.63 | 62.35 | 0.0041 | 3.02 | 0.120 |
| LEV | % of Methanol | 9 | 23.66 | 2.63 | 62.17 | 0.0071 | 0.60 | 0.579 |
| | pH of Buffer | 9 | 23.46 | 2.61 | 61.17 | 0.0050 | 1.13 | 0.380 |
| | Flow Rate | 9 | 23.68 | 2.63 | 62.28 | 0.0052 | 0.65 | 0.550 |
| MOX | % of Methanol | 9 | 24.31 | 2.70 | 65.68 | 0.0077 | 6.34 | 0.033 |
| | pH of Buffer | 9 | 24.25 | 2.69 | 65.35 | 0.0053 | 2.13 | 0.120 |
| | Flow Rate | 9 | 24.37 | 2.71 | 65.96 | 0.0087 | 10.38 | 0.010 |
| NOR | % of Methanol | 9 | 23.64 | 2.63 | 62.07 | 0.0033 | 1.00 | 0.420 |
| | pH of Buffer | 9 | 23.47 | 2.61 | 61.22 | 0.0058 | 2.66 | 0.150 |
| | Flow Rate | 9 | 23.67 | 2.63 | 62.23 | 0.0053 | 2.86 | 0.130 |
| OFL | % of Methanol | 9 | 23.67 | 2.63 | 62.23 | 0.0073 | 0.80 | 0.490 |
| | pH of Buffer | 9 | 23.54 | 2.62 | 61.59 | 0.0147 | 8.93 | 0.016 |
| | Flow Rate | 9 | 23.68 | 2.63 | 62.32 | 0.0072 | 6.07 | 0.040 |

**DISCUSSION:** In the reported research work, the RP-HPLC analytical method for the selected fluoroquinolones was developed and validated as per the ICH guidelines. Since all the validation parameters checked were within limits, the method was considered successfully validated.

By applying ANOVA as a form of statistical analysis for validating robustness, it was proved that any variation within the tested Design Space did not bring about any change in validity and effectiveness of the method. Since, the result was not significant at $p<0.01$, we accept the null

hypothesis that within this range there is no significant difference in the results, thereby proving that changes within the Design Space do not need revalidation of the method.

**CONCLUSION:** The developed RP–HPLC method was found to be suitable for the analysis of selected fluoroquinolones, namely, CIP, LEV, MOX, NOR, and OFL, in bulk form, as well as in marketed formulations like tablets. The method was found to be simple, quick, sensitive, economical, reliable, and precise. A Design Space was simultaneously created setting limits for critical attributes affecting the robustness. Any variations in parameters within the Design Space did not affect method performance, making the method easily transferrable without additional expenditure of time or money. This method can, therefore, be useful as a common and easily transferrable method for estimation of any of the selected fluoroquinolones, in bulk form or pharmaceutical dosage forms. This HPLC method will prove to be advantageous for laboratories handling numerous fluoroquinolones, especially since the method may be transferred without the need for revalidation within the Design Space.

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RESEARCH ARTICLE

# QbD Based Development of Two RP-HPLC Methods for Levofloxacin and Its Acid Degradation Product - Through Creation of Design Space for Critical Attributes and Application of ANOVA

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## ABSTRACT

Fluoroquinolones are antibacterials that are widely used in many countries, however there are growing worries about their presence in soil and water. Analytical methods for these medications are continually needed not only in the pharmaceutical area, but also in other fields such as chemical engineering and environmental sciences. Quality by Design (QbD) is a concept that ensures the robustness and adaptability of an analytical method or manufacturing process to any instrument, laboratory, or application. The major goal was to develop some easy stability indicating analytical procedures for Levofloxacin, a fluoroquinolone member, in the presence of its acid degradation product. The goal was to create a Reversed Phase-High Performance Liquid Chromatographic technology that could be used in any business and simply changed to suit their needs. The RP-HPLC procedures provided here employ a C18 column with dimensions of 25 cm (length) 0.46 mm (i.d.) and a particle size of 5 m. The first approach employed a 43:57 ratio of methanol and phosphate buffer (pH 3.0) as the mobile phase, while the second method used a 15:85 ratio of acetonitrile and 0.1 percent triethylamine (pH 3.0). The analytes were detected using a PDA detector wavelength of 294nm in both procedures, which were performed at 0.8 ml/min flow. To validate parameters, ICH-recommended validation standards were used, as well as statistical methodologies such as ANOVA and the creation of a Design Space to assign limiting values for the essential variables impacting robustness. Two simple and reliable RP–HPLC techniques were devised, one of which could be modified for LC–MS compatibility.

**Keywords:** RP-HPLC, QbD, Critical Attributes, Design Space, LC-MS, Levofloxacin

# INTRODUCTION

Antibacterials belonging to the fluoroquinolone class are commonly used to treat respiratory and urinary tract infections (FQs). According to the literature, the first quinolone was discovered as a by-product of the synthesis of chloroquine in the 1960s, and when this chemical was shown to have antibacterial action, it led to the development of nalidixic acid [1].Despite the passage of time and the introduction of newer members, older medications are still being utilised to treat a variety of infections [2-6].Quality by Design is a method of incorporating quality into a product or process from the beginning of the design or planning process. This approach is gaining traction in the pharmaceutical industry, owing to the fact that analytical techniques developed using QbD principles do not require redevelopment, revalidation, or reapproval after transfers, as long as the Design Space boundaries are not exceeded. This saves both time and money [7-11]. There are growing worries about these medications being found in soil and water as a result of their widespread and indiscriminate usage, stressing the need to find avenues to speed biodegradation. This highlights the need for novel and easy analytical methods that can be quickly adopted and implemented in a variety of sectors, including chemical engineering and environmental sciences. We attempted to develop a common HPLC approach for several fluoroquinolones previously, however the method failed to detect any degradants (12).Our efforts in developing and verifying two stability-indicating RP-HPLC techniques for the selected FQ, levofloxacin (LEV), while it is present alongside its acid degradant, LDA, acquired through forced degradation of LEV, are described in this paper. Figure 1 depicts the chemical structure of LEV.

A review of the literature revealed that there is no straightforward technique for this medicine. The majority of the described analytical approaches required simultaneous estimate of FQs, which made the method difficult [13-27].Some used gradient elution and fluorescence, as well as other advanced detection and quantification techniques [28-45].As a result, it was decided to design basic and quick stability signalling techniques that could be simply adapted for the bulk of the class. To design the techniques and validate the important parameters, ICH-specified guidelines and QbD principles were used [46-52].

## MATERIALS AND PROCEDURES

### Chemicals

Merck's methanol and acetonitrile (HPLC grade) were used. HPLC grade water, orthophosphoric acid and triethyl amine (HPLC grade of Merck), formic acid, and disodium hydrogen phosphate were all prepared in the lab using the BioAge Ultra Water Purification system or purchased from Merck (AR grade). Abaris Healthcare Pvt. Ltd., Ahmedabad, Gujarat, India, donated the pure medicine. The experiments were carried out at Goa College of Pharmacy's Central Instrument Laboratory in Panaji, Goa, India.

### Instrumentation

The following equipment/instruments were used: a Wensar electronic weighing balance, a Citizen sonicator, a Labtronics pH metre, a Jasco HPLC with ChromNav software, and a Phenomenex C18 column.

### Chromatographic Circumstances

Jasco CO-4061 HPLC system with Autosampler (AS-4050), PDA detector, and built-in degasser was employed. ChromNav software was utilised in the system .Phenomenex C18 column (25cm length, 0.46cm internal diameter, and 5 particle size of packing) was chosen after several testing. Varied solvents in various amounts were explored, with methanol with phosphate buffer and acetonitrile with triethylamine being the two compositions chosen. Method 1's mobile phase consisted of 43 parts methanol and 57 parts buffer. Dissolving disodium hydrogen phosphate in water and correcting the pH to 3.0 with orthophosphoric acid yielded phosphate buffer pH 3.0.
Method 2 utilised 15 parts acetonitrile and 85 parts 0.1 percent triethylamine (TEA) adjusted to pH 3.0 with formic acid as the mobile phase. Prior to use, the mobile phase components were sonicated for 15 minutes and filtered using

0.45 membrane filters. The flow rate was kept constant at 0.8mL/min for both procedures, and the detector was set to 294nm wavelength. The autosampler employed has a variable loop volume of 0-100L, and 20L was injected in this approach. Table 1 shows the optimal chromatographic settings for a certain fluoroquinolone.

## Development of a Method

### Mobile Phase Selection and Preparation

Mobile phases including methanol, acetonitrile, water, and buffers at various pH levels were tested in various quantities and flow rates[53-55].At flow rates of 0.8mL/min, satisfactory peaks were achieved using the mobile phases listed in Table 1 and setting the pH to 3.0 using a pH metre. Before being introduced into the system, both components of the mobile phase were vacuum filtered through 0.45m membrane filters and sonicated for 15 minutes.

### Standard Stock Solution Preparation

Depending on the procedure, the standard solutions of the medicines were produced in methanol/acetonitrile. To make standard stock solutions of 1000 g/mL of each medication, a quantity of 25 mg of each drug was weighed and dissolved in methanol/acetonitrile in 25mL volumetric flasks. To produce the appropriate concentrations of each medication, the standard stock solutions were further diluted with methanol/acetonitrile.

### Calibration Curve Preparation

Each drug's standard stock solution was transferred to a 10 mL volumetric flask and diluted to the desired concentration using methanol/acetonitrile. Aliquots were taken in such a way that the final concentrations were within the acceptable limit. Using optimal circumstances, three injections of 20 L of each concentration were examined. The average or mean of three readings was used to record each reading or peak area. For each medication, calibration curves were created by graphing peak areas recorded for each concentration on the y-axis and the drug concentration on the x-axis. For each drug's calibration curve, the coefficient of determination (R2) was computed.

### Experiments on Forced Degradation

Stock solutions of the medication LEV were produced in methanol/acetonitrile at a concentration of 1000 g/mL (1 mg/mL).2mL aliquots were collected from the stock and used to make a 10mL volume of 5N HCl. For 6 hours, these acidic solutions were placed in a water bath at 65°C.The deteriorated solutions were then neutralised and diluted with water to make up to 25mL.From these damaged samples, 1mL was taken and built up to 10mL with methanol/acetonitrile to inject as a sample[56-61].

## Validation of the Method

The method was validated using ICH guidelines, which included evaluating factors like accuracy, precision, linearity, robustness, ruggedness, detection, and quantification limits[62-71].When evaluating the results, acceptable limits for Relative Standard Deviation were set at less than 2%. (RSD).ANOVA was also utilised to prove the precision and robustness of the approach statistically.

## Perfection

For each of the medications, the precision of the devised approach was confirmed. Actual investigation of six replicate injections of a standard concentration of each medication yielded the peak areas [72].The RSD was used to assess the method's precision in terms of intra- and inter-day fluctuation in the peak areas. ANOVA was also used to establish the method's precision.

### Precision

For each of the drugs, the method's accuracy was tested by spiking a known concentration of each drug at three different concentration levels, namely 80%, 100%, and 1200%, and then comparing the difference between the expected/ theoretical value and the concentration actually determined by the method [73-75].

### Linearity

For the medication LEV, stock solutions of 1000 g/mL in methanol/acetonitrile were produced. Working standard solutions in the appropriate range were generated from these stocks and injected into the HPLC apparatus. Each medicine has been shown to be linear in the specified range. The calibration graph (obtained by plotting the drug's peak regions versus its concentration) was made using replicate analyses at all concentration levels, and the linearity of the connection was determined using the Microsoft Excel® application.

### Designing Critical Attributes with Robustness and a QbD Approach

Variation in crucial parameters, such as flow rate, pH, and fraction of mobile phase components, was used to confirm the robustness of the devised technique for chosen fluoroquinolones. ANOVA was used to verify robustness statistically [73,74,76].

### Limits of Detection and Quantification

The linearity curve was used to calculate the limit of detection (LOD) and limit of quantification (LOQ).These values were calculated using the following formulas: LOD=3.3/s
LOQ=10/s, where s is the slope of the calibration curve and is the standard deviation of the y-intercept of the regression line.

### LEV Calibration Curve

Table 3 shows the correlation coefficients (R2) and linearity equations for each of the methodologies under discussion.

### Validation of the Method

The procedure was verified and applied to commercially available formulations.

### Determining Critical Attributes and Their Scope

The important attributes determining the approach's effectiveness were identified and used to build a method that is both resilient and easy to transfer. These factors were then tested over a variety of ranges, as shown in Table 5.

### The Use of ANOVA in the Design of Design Space

It was discovered that the approaches were both robust when using ANOVA as a statistical analysis tool. As shown in Table 6, this exercise also resulted in the creation of a Design Space for each essential variable The f-ratio demonstrates that there is no substantial difference in efficiency between the two methods in the event of any planned or unintentional alteration in any of the important parameters within the Design Space.

## DISCUSSION

The new RP-HPLC techniques for the drug LEV in the presence of its acid degradant were developed and verified according to ICH guidelines in the study reported. It was demonstrated that any alteration within the examined Design Space had no effect on the validity and effectiveness of approaches using ANOVA as a form of statistical analysis for validating robustness. We accept the null hypothesis that there is no significant difference in the outcomes within this range because the result was not significant at p 0.05, indicating that modifications within the Design Space do not require revalidation of the method.

## CONCLUSION

The developed RP–HPLC methods were proven to be suitable for analysing the fluoroquinolone LEV in both bulk and marketed forms such as tablets. Simple, rapid, sensitive, inexpensive, dependable, and precise procedures were discovered. Method 2 can also be altered for use with LC-MS. Simultaneously, a Design Space was constructed, which defined limitations for essential attributes determining robustness. Variations in parameters inside the Design Space have no effect on the method's performance, allowing it to be easily transferred without additional effort or expense. Both of these approaches can be used to estimate LEV in the presence of its acid breakdown product, whether in bulk or in medicinal dosage forms. Method 2 using acetonitrile is also compatible with LC-MS, which is a benefit over Method 1.

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**Table 1: Chromatographic Conditions That Have Been Optimized**

| **Parameters** | **Method 1** | **Method 2** |
|---|---|---|
| Stationary phase (column) | C18 (250 mm×4.6 mm, 5 μm) | C18 (250 mm×4.6 mm, 5 μm) |
| Mobile phase | Methanol: 20mM phosphate buffer, pH 3.0 with Orthophosphoric acid | ACN: 0.1% TEA, pH 3.0 with Formic acid |
| Ratio (v/v) | 43:57 | 15:85 |
| Flow rate (mL/min) | 0.8 | 0.8 |
| Run time (min) | 8.0 | 10.0 |

| | | |
|---|---|---|
| Injection Volume (µL) | 20 | 20 |
| Detection Wavelength (nm) | 294 | 294 |

**Table 2: Shows The Methods Used To Determine The Efficiency Of A System.**

| Sr No | Parameters | Acceptance Criteria | Method 1 | | Method 2 | |
|---|---|---|---|---|---|---|
| | | | LEV | LDA | LEV | LDA |
| 1 | Plates of theorem | >1000 | 4517 | 4190 | 7642 | 7594 |
| 2 | a restraining element | <2 | 1.296 | 1.362 | 1.243 | 1.522 |
| 3 | RSD in the area | <2% | 0.528 | 0.616 | 1.861 | 1.155 |
| 4 | Retention Time RSD | <1% | 0.488 | 0.356 | 0.255 | 0.093 |
| 5 | Between-peak resolution | >2 | ____ | 2.169 | ____ | 12.000 |

**Table 3: Shows the Correlation Coefficients (R2) and Linearity Equations for Each of the Methodologies under Discussion.**

| Sr.No. | Drug | $R^2$ | Linearity equation |
|---|---|---|---|
| **1** | Method 1 | 0.999 | y = 121479x + 6584.9 |
| **2** | Method 2 | 0.999 | y = 21142x – 49348 |

**Table 4 : Summarises The Findings.**

| Parameter | Method 1 | Method 2 |
|---|---|---|
| Linearity range(µg/mL) | 30-210 | 10-120 |
| LOD(µg/mL) | 5.92 | 1.29 |
| LOQ(µg/mL) | 19.75 | 4.29 |
| Precision | Complies | Complies |
| Assay (Limits 90-110%) * | 98.71% | 98.71% |
| Accuracy | 95-101% | 100-101% |
| Robustness** | Complies | Complies |

**Table 5: Critical Attributes And Ranges Analyzed.**

| Sr.No. | Method 1 | | | Method 2 | | |
|---|---|---|---|---|---|---|
| | Critical Attribute | Value | Range | Critical Attribute | Value | Range |
| 1 | % of Methanol | 43% | 41-45% | % of Acetonitrile | 15% | 13-17% |
| 2 | pH of Buffer | 3.0 | 2.8-3.2 | pH of Buffer | 3.0 | 2.8-3.2 |
| 3 | Flow Rate (mL/min) | 0.8 | 0.6-1.0 | Flow Rate (mL/min) | 0.8 | 0.6-1.0 |

**Table 6 : The Approaches Were Both Robust When Using ANOVA As A Statistical Analysis Tool**

| Method | CA | n | Mean Conc (µg/mL) | *F obtained* | Critical F-value (0.05 significance level) |
|---|---|---|---|---|---|
| 1 (using concentration 60 µg/mL) | % of Methanol | 9 | 59.984 | 2.278 | 5.14 |
| | pH of Buffer | 9 | 59.911 | 2.182 | |
| | Flow Rate | 9 | 59.696 | 1.915 | |
| 2 (using concentration 25 µg/mL) | % of Acetonitrile | 9 | 24.836 | 0.529 | 5.14 |
| | pH of Buffer | 9 | 24.841 | 0.788 | |
| | Flow Rate | 9 | 24.873 | 0.383 | |

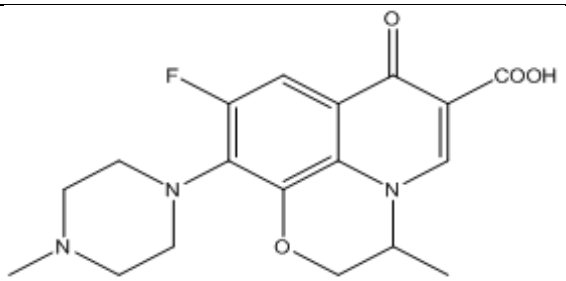


**Figure 1: shows the LEV's structure.**

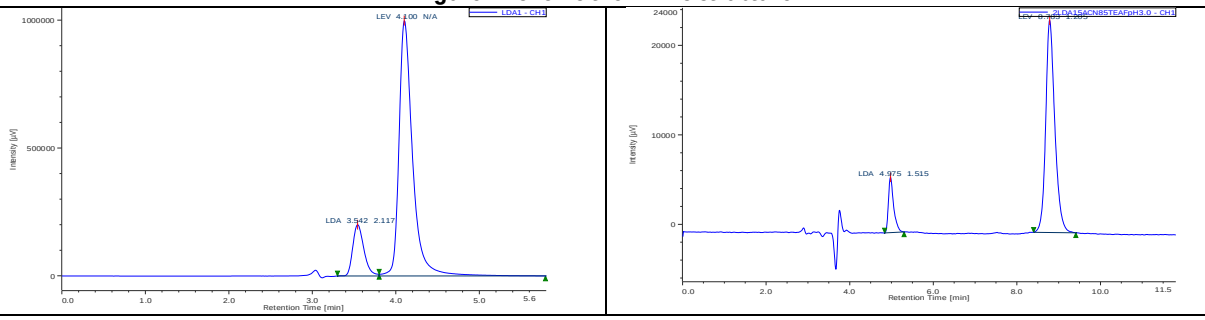


**Fig. 2: Representative LEV and Acid Degradant LDA chromatograms obtained using (a) Method 1 and (b) Method 2 5.**

# Certificate for Poster Presentation

70th IPC 2018
INDIAN PHARMACEUTICAL CONGRESS
December 21-23, 2018 - Delhi NCR

F-16

Theme
Pharma vision 2030

**Indian Pharmaceutical Congress Association**
hereby declares that

**Dr / Mr / Ms** **SACHI KUDCHADKAR**

has presented a paper entitled

**QBD BASED ANALYTICAL METHOD DEVELOPMENT THROUGH CREATION OF DESIGN SPACE FOR CRITICAL ATTRIBUTES – APPLICATION ON RP-HPLC METHOD DEVELOPMENT FOR FIVE FLUOROQUINOLONE ANTIBACTERIALS**

in the Scientific Session during 70th IPC 2018

**Theme :**
**Pharma Vision 2030 :**
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***held on December 1-2, 2021 at Goa College of Pharmacy, Panaji-Goa***

***Umesh Banakar, Ph.D.***
*Professor*

***Dr. Gopal Krishna Rao***
*Principal*

# ERRATA

| Sr. No. | Location | Error | To be read as / corrected to |
|---|---|---|---|
| 1 | p 8, line 1 | ie | i.e. |
| 2 | pp 26,27 MOX HPLC methods | terabutyl | tetrabutyl |
| 3 | p 30, OFL eye drops | sulpate | sulphate |
| 4 | p 43, LEV degradant | photodegradantn | photodegradant |
| 5 | p 45, NOR degradant | decarboxyated | decarboxylated |
| 6 | p 68, row 4 | dectection | detection |

## DECLARATION

I, Rupali Keny (Sachi Kudchadkar) hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any otherUniversity or Institution for the award of any research degree.

Place: Taleigao Plateau.

Date : 06-02-2023

Rupali Keny / Sachi Kudchadkar

## CERTIFICATE

I hereby certify that the work was carried out under my supervision and may be placed for evaluation.

(Guide)
Dr. Sanjay Pai P.N.
Professor and Head of Dept.,
Dept. of Pharmaceutical Analysis &
Pharmaceutical Chemistry,
Goa College of Pharmacy
Panaji – Goa.