SIMULTANEOUS ESTIMATION AND STABILITY STUDIES OF LOPERAMIDE HCL AND NORFLOXACIN IN COMBINED DOSAGE FORM BY REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the requirement of the Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

April-2012

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

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Submitted by
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CERTIFICATE

This is to certify that, the work embodied in the thesis entitled **"SIMULTANEOUS ESTIMATION AND STABILITY STUDIES OF LOPERAMIDE HCL AND NORFLOXACIN IN COMBINED DOSAGE FORM BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY"** is a bonafide research work carried out by Mr. Arun Kumar.G.R **(Reg. No: 26107222),** Student in Master of Pharmacy, Department of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, under my supervision and guidance during the academic year 2011-2012.

Date:
Place:

Prof. Dr. A. Rajasekaran, M. Pharm, Ph.D.,
Guide cum Principal

DECLARATION

I do here by declare that, the dissertation work entitled **"SIMULTANEOUS ESTIMATION AND STABILITY STUDIES OF LOPERAMIDE HCL AND NORFLOXACIN IN COMBINED DOSAGE FORM BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY"** being submitted for the partial fulfillment of Master of Pharmacy in Pharmaceutical Analysis for the academic year 2011-2012 of KMCH. College of Pharmacy affiliated to The Tamilnadu Dr. M.G.R. Medical University carried out under the guidance of **Prof. Dr. A. Rajasekaran, M. Pharm, Ph.D.,** at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore and co-guided by Mr.Y.M.Tiwary, senior executive, Wockhardt ltd.

I abide that all the data presented in this report will be treated with utmost confidentiality.

Date:

Place: **Arun Kumar. G.R**

EVALUATION CERTIFICATE

This is to certify that, the work embodied in the thesis entitled **"SIMULTANEOUS ESTIMATION AND STABILITY STUDIES OF LOPERAMIDE HCL AND NORFLOXACIN IN COMBINED DOSAGE FORM BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY"** submitted by Mr. Arun Kumar.G.R **(Reg. No: 26107222),** to The Tamilnadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy**, in **Pharmaceutical Analysis**, is a bonafide research work carried out by the candidate at K.M.C.H. College of Pharmacy and wockhardt ltd was evaluated by us during academic year 2011-2012.

Examination Center: KMCH College of Pharmacy, Coimbatore.

Date:

Internal Examiner **External Examiner**

Convener of Examinations

ACKNOWLEDGEMENT

First and foremost I extremely beholden to my esteemed guide, **Prof. Dr. A. Rajasekaran, M.Pharm., Ph.D., Principal,** Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore, for his constant insight, personal advice, countless serenity and pain taking effort in all stages of this study.

It gives me immense pleasure to record my deep sense of gratitude and indebtedness to my co-guide **Mr.Y.M.Tiwary, Senior executive, Wockhardt Ltd.,**for his personal advice, effort, moral support and encouragement during the course of my work under his guidance, I was able to improve my scientific approach and outlook throughout this work.

I extend my profound thanks and gratitude to **Mr.Pradeep Redkar**, **manager -Quality control Wockhardt Ltd.,**

I owe a profound depth of gratitude to **Mr. J. Dharuman, M.Pharm**. Asst. Professor, Head of the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore, for his constant valuable guidance help and advice during the course of my work.

I take this opportunity to thank **Mr. K. Suresh Kumar M.Pharm., (Ph.D).,** **and Mr. I. Ponnilavarasan M.Pharm., (Ph.D).,** Asst. Professor, Department of Pharmaceutical Analysis, KMCH College of Pharmacy for providing valuable ideas to perform my dissertation work.

My respectful regards to our beloved Managing trustee **Dr. Thavamani D. Palanisamy** and our respected Chairman **Dr. Nalla. G. Palanisamy,** KMCH College of Pharmacy, Coimbatore.

I take this opportunity with pride and immense pleasure to thank all my **Teachers** who lit the light of knowledge and wisdom in my life.

I express my hearty thanks to all my classmates and friends for their timely help and co-operation during the course of my dissertation work.

I express my hearty thanks to **Mr. Manoj Sharma, Mr.Chandan Parihar, Mr.Gautam Saini, Wockhardt Ltd.,** for their help during the course of my dissertation work.

I acknowledge with gratitude, the memorable company, co-operation, support and prayers offered to me by my dear most friends for their prayers and constant support.

Above all I dedicate myself before the unfailing presence of **God** and constant love and encouragement given to me by my beloved **Mother, Father, and Brother** who deserves the credit of success in whatever work I did.

Last but not least, I would like to thanks everyone who was important to the successful realization of the thesis; as well apologize that I could not mention personally

.

Arun kumar G.R

ABBREVIATIONS

HPLC	:	High performance liquid chromatography
UV	:	Ultra violet
ICH	:	International conference on harmonization
SD	:	Standard deviation
RSD	:	Relative standard deviation
UV	:	Ultra Violet
PDA	:	Photo diode array
ACN	:	Acetonitrile
H_3PO_4	:	Phosphoric acid
NOR	:	Norfloxacin
LOP	:	Loperamide Hcl
nm	:	Nanometer
mg	:	Milligram
µg	:	Microgram
PA	:	Purity Angle
TH	:	Purity threshold
k	:	Retention time
g	:	gram
v/v	:	Volume by Volume
µg/ml	:	Microgram per milliliter
pH	:	Hydrogen ion concentration
ml	:	Milliliter
µg	:	Microgram

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ABSTRACT

A simple, precise, accurate RP-HPLC method has been developed and validated for the analysis and stability studies of Norfloxacin (NOR) and Loperamide (LOP) in combined dosage form. The chromatographic separation and quantization of NOR and LOP in HPLC was achieved on a Phenomenex C-18(250 x 4.6 mm 5 µm) column using the mobile phase in isocratic mode constituting of eluent ACN: Water: H_3PO_4 (pH adjusted to 4.5 with triethylamine) in the ratio 50:50:0.2 v/v/v at a flow rate of 1 ml/min. Eluted components were detected at 222 nm. The method showed high sensitivity and good linearity over the concentration range of 5 to 25 µg/ml, Y=32737 x – 4790, Y=24054 x + 3766 and the correlation coefficient R^2 was found to be 0.9998, 0.9995 for NOR and LOP respectively. The retention time was found to be 2.09 for NOR and 4.51 for LOP. The method validation was carried out as per ICH guidelines. The LOD and LOQ were found to be 1.17 µg/ml and 3.59 µg/ml for NOR and 0.16 µg/ml and 0.39 µg/ml for LOP respectively. The mean recovery values for NOR and LOP was found to be in the range of 99.46 – 100.76 %. The method was specific and no interference was observed when the drugs were estimated in presence of excipients. The method was validated for linearity, range, precision, accuracy, ruggedness and robustness. After the validation the method was successfully applied for the estimation of norfloxacin and loperamide in combined dosage form.

CHAPTER 1

INTRODUCTION

As the mankind made his way through remote times and places, he was always followed by disease and sickness from ill health. Market is filled with combination of drugs in various dosage forms. The multi-component formulations are so much important nowadays due to greater patient acceptability, increased potency, multiple action, fewer side effects and quicker relief.

Analytical chemistry is the science of making quantitative measurements. In practice, quantifying analytes in a complex sample becomes an exercise in problem solving.

To meet these needs, analytical chemistry courses usually emphasize equilibrium, spectroscopic and electrochemical analysis, separations and statistics. Analytical chemistry requires broad background knowledge of chemical and physical concepts. These hypermedia documents contain links to the fundamental principles that underlay the different analytical methods. A fundamental understanding makes it easier to identify when a particular problem cannot be solved by traditional methods, and gives an analyst the knowledge that is needed to develop creative approaches or new analytical methods[1].

General analytical techniques: [2-3]

The main techniques used for quantitative analysis are based upon:

1) Appropriate chemical reaction based the amount of reagent required for the entire reactions and the total quantity of product obtained.
2) Suitable electrical measurements, involves the measurement of current, voltage or resistance as a measure to the concentration of a certain analyte present.
3) The measurement of optical properties which depends either on measurement of the radiant energy absorbed or the emission of radiant energy of a particular wavelength by the sample.
4) In the area of pharmaceutical science and technology instrumental analysis have become an important mean of quantitative and qualitative estimation. Due to the greater sensitivity, speed of analysis, low detection limit, ability for simultaneous determination and programmed operational availability of

the instruments as compared with the other method of analysis have shows its prevalence. Most commonly used instrumental methods are Chromatography, Ultra violet spectroscopy, Infra red spectroscopy, X-ray methods etc.

5) Radioactivity is the measurement of concentration of the radiation from a naturally radioactive substances or an induced radioactive substance produced from the exposure of the test compound to a neutral source.
6) In case of thermal methods alteration in weight or alteration in power, calculated as a function of temperature is considered. Different types of thermal methods commonly used are thermo gravimetry, differential scanning calorimetry etc.

From all the methods mention above for the analysis of pharmaceutical chromatography has improved a lot for both the quantitative and qualitative analysis. Different types of chromatographic methods are available.

Chromatographic methods:

- Gas chromatography (GC).
- High performance liquid chromatography (HPLC).
- Liquid chromatography-mass spectroscopy (LC-MS).
- Gas chromatography –mass spectroscopy (GC-MS).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHHY:

High Performance Liquid Chromatography (HPLC) is a type of chromatography which is predominantly used analytical techniques. Chromatographic method is a separation technique which involves movement of analyte between the stationary phase and mobile phase.

Instrumentation HPLC system: [4]

HPLC instrumentation includes a

- Mobile phase reservoir
- Pump
- Injector
- Column
- Detector
- Data system.

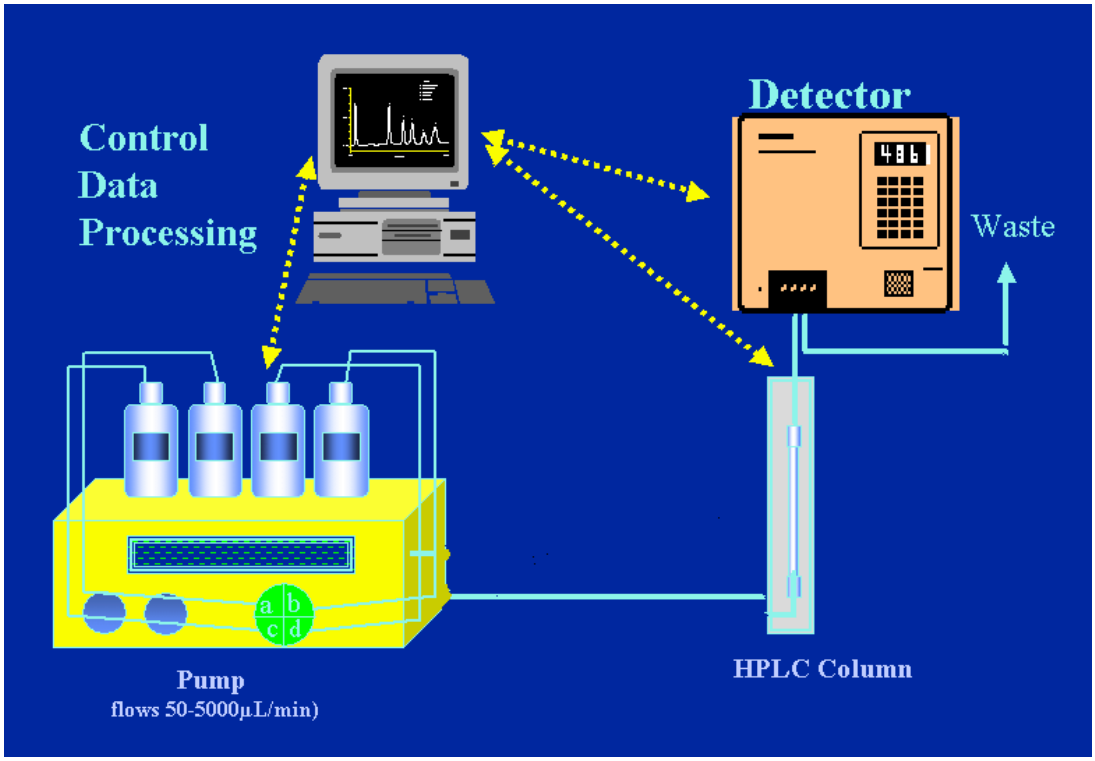

Fig 1. Schematic representation of HPLC instrument

Mobile phase reservoir:

The commonly used solvent reservoir is a glass bottle. Most of the manufacturers provide bottles with special caps. For connection of this mobile phase reservoir to the pump inlet Teflon tubing and filters are used.

Pump:

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressure. There are many advantages to using smaller particles, but they are not essential for all separations. The advantages are higher resolution, faster analyses, and increased sample load capacity.

Injector:

Sample can be introduced in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors.

Column:

HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 µm) particles. The internal diameter of the columns is usually 4.6 mm.

This is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution.

Detector:

When a substance has passed through the column there are several ways to detect it. A common method which is easy to explain is ultra-violet absorption. The amount of light absorbed will depend on the amount of a particular compound that passes through the beam at the time.[5]

Data system:

Modern data collection techniques can aid the signal analysis since the detector signal is electronic. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time. The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention.

ADVANTAGES OF HPLC: [6]

1. Wide variety of packing materials allows the separation of most chemical species. The phases that are most extensively used for drug substances of low molecular weight (less than 1000) and their decomposition products or metabolites are the adsorption systems based on silica and the reversed –phase systems based on octyl silyl or octa decyl silyl bonded on silica.
2. The different types of detectors available permit the sensitive detection of most chemical type, and the accuracy and precision with which eluted substances may be quantified.
3. Micro particulate packing materials give excellent separation of similar substances.
4. The number of theoretical plates given by a standard analytical column is of the order of 5000-10000 and this gives adequate resolution of the vast majority of mixtures which are likely to require separation.
5. A combination of HPLC and spectrometric techniques allows the simultaneous quantization and identification of solutes as they elute from the column.
6. In the pharmaceutical industry, it is essential to produce pure drug substances, suitable for human consumption for in a cost effective manner. The purity of drug substance can be checked by separation techniques such a GC, TLC,

HPLC. These techniques tends to be more sensitive and specific than spectroscopic method.[7]

7. HPLC has an advantage over GC as an analytical technique, since analytes need be neither volatile nor extremely stable to elevent temperatures. Highly accurate, almost universal detectors, makes quantification easier than with TLC.
8. Standardization either by external or internal standard techniques made it possible to convert for structurally depended differences in detector response.[8]

ANALYTICAL METHOD DEVELOPMENT:

Analytical method development plays an important role in the discovery, development and manufacture of pharmaceuticals. The official test methods that results from these process are used by quality control laboratories to ensure that results from these process are used by quality control laboratories to ensure the identity, potency, purity and performance of drug products.[9] To ensure compliance with quality and safety standards USA, Europe, Japan, and other countries have published their own pharmacopeia's.

Analytical Method Development by HPLC:

High performance liquid chromatography (HPLC) is widely used technique in various industries such as pharmaceuticals, nutraceutical, cosmeceutical, oleochemical, biotechnological, food and beverage industries. HPLC is uded to analyze raw materials and products during formulation and quality control assurance stages. In HPLC the separation is based on interaction and differential partition of the sample between the mobile pahse and stationary phase[10.]

Modes of HPLC:

There are five modes in HPLC [11]

LC Mode	Stationary phase	Mobile phase	Principle
Normal phase Chromatography	Silica gel	n-Hexane/Isopropyl alcohol	Adsorption
Reverse phase Chromatography	Silica C18(ODS)	Methanol/water	Partition
Sizeexclusion chromatography	Porous polymer	THF	Gel permeation
Ion-exchange chromatography	Ion exchange gel	Buffer solution	Ion exchange
Affinity chromatography	Packing with ligand	Buffer solution	Affinity

Normal Phase Chromatography:[12]

Normal phase chromatography is a chromatographic technique that uses organic solvents as mobile phase and a polar stationary phase. Here the less polar components elute faster than the more polar components. Although it is described as "normal" it is not the most commonly used form of HPLC. The column is filled with tiny silica particles and is non- polar. A typical column has an internal diameter of 4.6mm. polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds. The non-polar ones will therefore pass more quickly through the column.

Reversed —Phase Chromatography:

RP-HPLC method is most commonly used in the pharmaceutical analysis as the compounds elute faster with high resolution. UV detection is the most common detection technique. Reverse phase chromatography, a bonded phase chromatography technique, uses water as the base solvent. Separation based on solvent strength and selectivity also may affected by column temperature and pH. In general the more polar components elute faster than less polar components.

Chromatogram

All serous steps in method development will be summarized and a sequence of events that necessary to develop the method efficiently. The steps will be discussed in the same oder as they would be investigated during the method development process. The rational will be illustrated by focussing on developing a stability-indicating HPLC- UV method. The principles, however will be applicable to most other HPLC methods. High performance liquid chromatography is a convenient separation technique used for wide type of samples, due to its exceptional resolving power, speed and nano molecular detection levels.

It is used in pharmaceutical research and development

- To purify synthetic or natural products.
- To characterize metabolites.
- To assay active ingredients, impurities, degradation products and in dissolution.
- In pharmacodynamics and pharmacokinetic studies.

Analytical method validation:

The validation of analytical procedures is an important part of the registration for application of a new drug[13-22]. The international conference on the Harmonization of technical requirements for registration of pharmaceutical for human used (ICH) has harmonised the requirements in two guidelines. Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desire result[23-24].

Method validation is a process of demonstrating that analytical procedures are suitable for their intended use and they support the identity, quality. Purity and potency of the drug substance and drug products. A successful validation guarantees that both the technical and regulatory objectives of the analytical method have been fulfilled. The real goal of validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method[25-26].

Importance Method Validation

The quality of analytical data is a key factor in the success of a drug development program. The process of method development and validation has a direct impact on the quality of these data[27].

Analytical validation is a very important feature of any package of information submitted to international regulatory agencies in support of new product marketing or clinical trials applications. A thorough method development can almost rule out all potential problems, at the same time, a thorough validation program can address the most common ones and provide assurance for the intended purpose. In other words, a thorough validation can fulfil all the technical and regulatory objectives. A direct consequence and most significant outcome from any method validation exercise is the development of meaningful specifications, which can be predicted upon the use of validated analytical procedures that can assess changes in a drug substance or drug product during its life time. Analytical characteristics listed below may not be applicable to every test procedure or every particular material. It usually depends on the purpose for which the procedure is required; however, these aspects of validation should be given importance[28].

PARAMETERS OF ANALYTICAL METHOD VALIDATION

1. Specificity: specificity is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response in test mixtures containing the analyte and all potential sample components is compared with the response of a solution containing only the analyte.

2. Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. In a method with high accuracy, a sample is analyzed and the measured value should ideally be identical to the true value. Accuracy is represented and determined by recovery experiments. The standard range being 10% above or below the estimated range of claim. For assay methods, spiked samples are prepared in triplicate at three levels across a range of 50-150% of the target concentration. The percent recovery should then be calculated.

3. Precision: The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Precision may be considered at three levels:

Repeatability

Intermediate precision

Reproducibility

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

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

3.3. Reproducibility: Reproducibility expresses the precision between laboratories (collaborative studies, usually applied for standardization of methodology).

4. Limits of detection and quantification: The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. It is expressed as a concentration at a specified signal: noise ratio, usually 3:1. The limit of quantization (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The ICH has recommended a signal: noise ratio 10:1. LOD and LOQ may also be calculated based on the Standard deviation of the response (SD) and the slope of the calibration curve(s) at levels approximating the LOD according to the formulae. The limit of detection (LOD) and limit of quantization (LOQ) may be expressed as:

$$LOD = 3.3\, a/S$$

$$LOQ = 10\, a/S$$

Where a = the standard deviation of the response

S = the slope of the calibration curve

5. Linearity: Linearity of an analytical procedure is its ability to obtain test result which is directly proportional to the concentration of the analyte in the sample.

6) Range: The range of the analytical procedure is the interval between the upper and lower concentration of the analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

7. Robustness: Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters. It also provides some indication of the reliability of an analytical method during normal usage. Parameters that should be investigated are percent organic content in the mobile phase, pH of the mobile phase, buffer concentration, temperature, and injection volume. These parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment[29, 30].

8. **Ruggedness**: It involves analyzing the same sample under same experimental conditions by different analysts to check reproducibility of the method[31].

9. System suitability: System suitability of method was performed by calculating the chromatographic parameters like column efficiency, resolution, and asymmetric factor on the repetitive injection of standard solution[32].

Stability studies [33, 34]

The stability studies give an evidence for the variation of quality of drug product under the influence of various environmental factors such as temperature, humidity and light. The international conference of harmonization (ICH) has developed the guideline for stability of new drug substance and product. This guideline gives an idea about the stability data package required for registration.

Main aim of carrying out the stability studies was to determine the re-test of the drug substance, establishing the shelf life of the drug products and recommending the proper storage condition of the drug product for long term storage and use. As a whole the stability studies are very much essential because the physical, chemical and microbial variation may cause a high degree of impact on the efficiency and security of the final drug product.

New dosage forms

A new dosage form is defined as a medicinal product which contains the same active substance but in the form of different pharmaceutical product type as included in an existing product approved by the regulatory authority. This product may include product of new delivery system, various administration route, and different dosage form of same route of administration. The protocol for the new dosage forms stability studies may follow principles in the parent stability guideline. Mainly these studies include 6 months accelerated and 6 month long term data studies under the influence of various environmental factors.

Parameters for stability study

The international conference of harmonization has recommended various parameters for the stability testing of different pharmaceuticals. Mainly the parameters can be classified into three categories like physical, chemical, and microbiological.

Physical parameters: The physical changes may be important criteria for stability testing. Mainly appearance, melting point, clarity and colour of the solution, moisture content or water content and particle size are checked and reported. All these factors must be under the acceptance range.

Chemical parameters: This is one of the important areas where more concentration has to be taken in the time of stability testing. The determination of degradation products and the potency of the drug must be calculated by assay. Since these factors can affect the efficiency of the drug product proper testing must be required in this case.

Microbiological parameter: As because the drugs have to be indented for long term storage chances of growth of micro-organism are high. So proper testing must be done for verifying any microbial changes taking place as due to various environmental factors.

Batch selection:

For both the bulk drug and drug dosage form stability studies must be carried out on at least three batches with a minimum of 6 month duration for both accelerated and long term testing. Manufacturing of the drug substance must be done for pilot scale in

the same route and method of manufacturing that was done on a manufacturing scale. For the drug product, two of the tree product must be pilot scale, and the third may be smaller. The batches taken must processes should be similar to the marketed drug product manufacturing procedure and quality specification.

Storage condition:

The storage condition for the stability studies was developed by ICH and it was mainly based on four geographic regions or climate zones. The world geographical region was defined by four climatic zones I – IV. Long term testing for the drug product is normally for every 3 month for the first year, and for 6 month for every 6 month over the second year and then annually. For the accelerated testing for the drug product is normally for 1 month, 3 months and 6 months. A significant change in the stability for drug product is when the substance no longer meets the specification. For a drug product a significant change is considered when a 5 % change in potency, pH exceeds the limit, failure in dissolution limit and failure in physical attributes. For 12 month long term data drugs must be stored at a condition of 25^0 C /60%RH and for 6 month accelerated study drugs must be stored at a condition of 40^0C/75%RH.

CHAPTER 2
LITERATURE REVIEW

Nageswara Roa et al (2003) had reported a simple high performance liquid chromatographic method for the separation and estimation of synthetic impurities of norfloxacin in pharmaceutical dosage form. The developed method was used for the determination of chemical reactions during the development process an in the quality assurance department. Separation by the developed method was carried out by using a C18 column and the mobile phase used was 0.01M potassium dihydrogen orthophosphate and Acetonitrile at a ratio 60:40, v/v at pH 3.0.The detection wavelength was 260 nm diode array detector and the determination was done under isocratic condition at flow rate of 1.0ml/min. The developed method was found to be specific and precise for the estimation of raw material, intermediates and finished formulation of norfloxacin[35].

Breda Simonovska et al (1999) had evaluated and developed a high-performance thin-layer chromatography (HPTLC) method with direct fluorescence measurement for the estimation of norfloxacin. The validation of the developed method was carried out at a limit of 10 mg of norfloxacin per square meter on a stainless steel surface. Further modification of the method can be done at lower detection limit of about 5 ng norfloxacin. Comparison of the test solution were done between the developed high performance thin-layer chromatographic and the known HPLC method. Linearity, accuracy, precision were determined for the developed HPTLC method by densitometer or video system and was found to be almost comparable with the known HPLC method[36].

Kowalski et al (2005) developed a high performance liquid chromatography for the determination of norfloxacin from chicken tissues taken from different parts like liver, muscles and fat. The superannuated collected from tissue homogenate by centrifugation was used as the test sample. The analysis of the sample was done by using a reverse-phase C-18 column. The detection was done by using a fluorescence detector at 278 nm and 456 nm as excitation wavelength and emission wavelength respectively. The mobile phase used for the analysis was water: methanol at a ratio of

65:35v/v at a pH 3 adjusted with phosphoric acid, which contain tetrabutyl ammonium hydrogen sulphate as ion-pairing agent. The isocratic condition was carried out at a flow rate of 1ml/min. the limit of detection of norfloxacin in chicken tissues was found to be 2.5 ng/ml[37].

Fawas et al (1996) had carried out a high performance liquid chromatography for the determination of bioavailability of norfloxacin from PEG 6000 solid dispersion and cyclodextrin inclusion complexes in rabbits. The study was done in pure form of norfloxacin and its pharmaceutical formulation in various solvents form. The concentration of norfloxacin rabbit plasma was analysed by HPLC method with fluorimetric detection. The separation of the sample was achieved by using a Nucleosil C-18 column. The eluent used was a mixture of dihydrogenpotassium phosphate: methanol: Acetonitrile at ratio of 78:13:9 at Ph 3 adjusted with ortho-phosphoric acid. The flow rate set for the determination was 1.6 ml/min and the detection was carried out at 380 and 450 nm as excitation and emission wavelength respectively. From the data's obtained from the plasma concentration profiles it was found that the absorption of norfloxacin from SD and inclusion complexes is higher as compared with pure drug[38].

Bedore et al (2007) had developed a validated high performance liquid chromatography method with ultraviolet detection for norfloxacin from human plasma and application of the developed method for the bioequivalence study of two norfloxacin formulations. Ciprofloxacin was used as the internal standard for the study. Liquid-liquid extraction method was used for the extraction of norfloxacin and the internal standards from human plasma. Separation of norfloxacin, internal standard and plasma interferent were done by using a C-18 column with a mixture of 20mM sodium hydrogen phosphate buffer (pH 3.0) and Acetonitrile with a ratio of 88:12 v/v. The detection was carried out at a wavelength of 280 nm. The developed method was found to be linear from a range 25-3000 ng/ml. the recovery study and precision was performed and observed within the acceptable limits[39.]

Nafisur Rahman et al (2004) had reported a kinetic spectrometric method for the determination of norfloxacin in pharmaceutical dosage form. The development was done based on the oxidation of norfloxacin by alkaline potassium permanganate. The rate of change of absorbance is measured at 603 nm spectroscopically. The

calibration graph was made for determining the concentration of the drug by fixed time method. The linearity was observed at a range of 2.0 to 20 mg/ml. the obtained results are statically validated through recovery studies. The developed method was used for the estimation of norfloxacin in pharmaceutical dosage forms. The result finally obtained shows good accuracy and precision[40].

Ibrahim et al (2009) have developed a simple spectroscopic validated method for the estimation of norfloxacin in pharmaceutical dosage form. The coloured product formed was measured spectroscopically at a wavelength of 625 nm. The different factors affecting the reaction was observed and recorded. The reaction pathway was postulated. Calculation of the activation energy of reaction was calculated and it's found to be 5.072 kj/mol. The calibration curve was carried out by initial rate and fixed time method, and the graph were constructed at arrange of 20-150 and the limit of detection was found to be 8.4 and 3.2 gm/ml for initial rate and fixed method respectively. There was no interference observed from the excipients commonly found in the pharmaceutical dosage form. Interferences were observed from the excipients that are commonly present in the pharmaceutical formulations. The developed methods were totally validated and the results were found satisfactory. The developed method was successfully applied for the estimation of the norfloxacin in pharmaceutical dosage form[41].

Johnson et al (2001) used a high performance liquid chromatography method for the stability study of norfloxacin oral syrup at room temperature under refrigerated condition for 56 days. a waters HPLC instrument with absorbance detector and a variable volume injector. The stationary phased used was spherisorb phenyl column and separation was done by using a eluent as a mixture of monobasic potassium phosphate buffer, Acetonitrile, methanol and trifluroacetic acid at ratio of 80:15:5:0.3. The flow rate was 1.0 ml and UV detection was done at 278 nm. The stability-indicating method was done by using a sample of norfloxacin under heat, light and pH. The calibration curve was plotted between a range from 4.0 to 6.0 µg/ml. the correlation coefficient was found to be larger than 0.999. The precision was found to be satisfactory[42].

Al deeb et al (1995) had developed a HPLC method for the estimation for the norfloxacin in bulk form in tablets. A shimadzu liquid chromatography was used with

a UV detector and reodyne injector. The mobile phase used for the separation of norfloxacin was Acetonitrile, terebutyl ammonium hydroxide, phosphoric acid and water, with a flow rate of 2 ml/min and the detection was done at wavelength of 278 nm. The stability indicating method was done by accelerated photolysis[43].

Ivana et al (2008) had developed a RP-HPLC method for the quantitative estimation of loperamide from its acid degradation product. The separation from degradation product was achieved by using a c-18 column. The eluent used for the separation of loperamide from its degradation product was sodium-octansulphonate, triethyl amine and ammonium hydroxide in water: Acetonitrile at a ratio of 45:55. The pH of the eluent was adjusted to 3.2 with phosphoric acid. The linearity graph was plotted between a range of 10-100 µg/cm. the developed method was applied for the determination of the marketed pharmaceutical formulation. The degradation of loperamide was done by using hydrochloride acid solution at different temperature[44].

Streel et al (2004) had developed a liquid chromatography-tandem mass spectrometry for the analysis of loperamide in human plasma. The sample was separated by using methanol and ammonium acetate solution as mobile phase. A LC-MS/MS system with atmospheric pressure chemical ionization is used for the analysis of loperamide. The quantitation limit was found to be 50 ng/ml and further validation was also done for the newly developed method like recovery, precision, trueness, accuracy and linearity[45].

Maycock et al (2000) had developed a liquid chromatographic method for the estimation of loperamide in rat plasma and bovine serum solutions. The rat plasma solutions were treated with meta phosphoric acid with a intention of protein precipitation. The supernatant produced were used as the sample for analysis and were injected to a reverse phase C18 column and the detection was done by using a UV detector at a wavelength of 195 nm. Isocratic condition was employed for the separation and the flow rate was set at 1 ml/min at by using Acetonitrile: diethylamide: sodium phosphate buffer (40:0.08:60v/v/v) with 22 minutes as elution time. The method shows linearity between a range of 0-3 mg/ ml and 0-1 mg/ml for bovine serum solution and rat plasma solution respectively. The validation of the developed method was done and finally it was found to be suitable for the determination of bovine serum solution and rat plasma solution[46]

El Sherif et al (2000) had developed a two Spectrophotometric determination of loperamide in the presences of degradation products. The first method was based on ion-pair association complex formation by using different dyes like bromothymol blue, bromophenyl blue and naphtol blue black. Chloroform was used for the extraction of colored product and spectrophotometrically at 414, 415 and 627 nm for bromothymol blue, bromophenyl blue and naphtol blue black respectively. The method was found good for the analysis of loperamide degradation product. The developed second method was based between reactions of loperamide with iodine in chloroform. Linearity was observer at a range of 2.5–17.5 and 2.5–22.5 mg/ ml at 295 and 363 nm, respectively. The developed methods have been used for the analysis of the drug in pure form and in its pharmaceutical formulation[47].

Alastair et al (2000) had developed and reported a liquid chromatography- mass spectroscopy method for the determination of loperamide and its N- dimethyl metabolite in human plasma. Internal standard in the assay was *O*-Acetyl-loperamide. The samples were extracted with methyl tert.-butyl ether and the separation was achieved by a C18 as column and Acetonitrile –water containing 20nM ammonium acetate. No interfering peaks were detected at the time of separation by HPLC methods. Samples were analysed by using electro spray ionization in a triple quadrupole mass spectrometer. The method was satisfactorily validated[48].

Mahmoud et al (2011) had reported a RP-HPLC method for the simultaneous determination of tinidazole and norfloxacin in pharmaceutical tablet dosage form in the presence of some impurity. An isocratic condition was followed for the separation and the stationary phase used RP-18 column using the eluent methanol: 0.025M KH_2PO_4 with pH 3 adjusted with ortho-phosphoric acid. The flow rate was set at 4 ml/min and the detection wavelength was 290 nm. The linear calibration curve was constructed between a range of 1-80 µg/mL for two drugs. The method was satisfactorily validated as per ICH guideline, the newly developed method was found to be accurate and was successfully used for the estimation of norfloxacin and tinidazole in its dosage form[49].

Wang Jindong et al (1999) had developed a high performance liquid chromatography for the simultaneous estimation of norfloxacin and trimethorpimum. The method was developed by using a Zorbax ODS column as stationary phase and the separation was achieved by using eluent containing water: Acetonitrile: triethylamine at a ratio of 325:175:2 at pH 3.0 adjusted with phosphoric acid. The detection was carried out by using a UV detector at a wavelength of 240 nm and the flow rate was set at 1.0 ml/min. the linearity was observed between a range of 100-300 and 25-75 mg/L for norfloxacin and trimethopriumum respectively. The developed method was validated and found to be simple, sensitive and stable[50].

Argekar et al (2006) had reported a RP-HPLC method for the determination of norfloxacin and tinidazole in tablet dosage form. The method was developed by using a C-18 column as stationary phase and 0.2 % Triethylamine in water: Acetonitrile at a ratio of 80:20 with pH 2.6 to 2.8 adjusted with Phosphoric acid. The detector wavelength was set at 311 nm with a flow rate of 1 ml/min. the linearity was found to be in the range of 20-200µg/mL and 30 - 300 µg/mL for norfloxacin and tinidazole respectively[51].

Reddy et al (1999) had reported a simultaneous determination method for norfloxacin and tinidazole in pharmaceutical formulation by derivative Spectrophotometric and graphical absorbance ratio method. It was found that norfloxacin and tinidazole has an maximum absorbance at 275 and 317.8 nm respectively. The absorbance of norfloxacin was measured at 264.2 and for tinidazole at 306.2 nm which are the zero crossing point of tinidazole and norfloxacin respectively in derivative spectrophotometry. The linearity was observed at arrange of of 0-24 µg/ml for norfloxacin and 0-36 µg/ml for tinidazole. In the case of graphical absorbance ratio method the Absorbance was measured at 275 nm and 300.4 nm for each drug and the Beer's law is obeyed in the concentration range of 0-20 µg/ml for both drugs. The developed methods were validated and were found to be satisfactory[52].

Pinak Patel et al (1999) had developed a validated high performance liquid chromatography method for the simultaneous determination of norfloxacin and ornidazole. The method was developed by using a PRONTOSIL AQ ODS coloumn. Isocratic condition was carried out by using 50mM Sodium dihydrogen phosphate

buffer: ACN: Methanol at pH 2.5 adjusted with orthophosphoric acid. The elution rate was set at 1ml/min with a run time of 10 min and the detection was done at 294 nm. The developed method was found to be linear at arrange of 4-20 µg/ml and 5-25 µg/ml for norfloxacin and ornidazole respectively. The method was found to be accurate, precise and specific. The developed method was applied successfully for the simultaneous determination of norfloxacin and ornidazole in combined formulation[53].

CHAPTER 3

AIM AND OBJECTIVE

The number of drugs and drug formulations introduced into the market by pharmaceutical industries is rising at an alarming pace. These drugs and formulations may be either novel entities or partial modifications of the existing ones or they may be multi component dosage forms. The complexity of the dosage form posses' significant challenge to the analytical chemist during the development of assay methods.

Development of newer analytical procedures for the evaluation of drugs and their formulation is necessary for the following reasons.

1) The drug or drug combinations may not be official in pharmacopoeia.
2) A literature survey may not reveal any analytical procedure for the drug or its combinations.
3) Analytical methods for the quantification of the drug or its combination from biological fluids may not be available.
4) Newer methods are also suggested when the existing method are expensive, problematic and are not dependable and adequately sensitive.

The formulation consisting of Loperamide hydrochloride and Norfloxacin in combination is used in the treatment of diarrhea. For the determination of norfloxacin HPLC[35], HPTLC[36] and Spectroscopic method[40, 41] and for the determination of loperamide HPLC[44], LC-MS[45], Spectroscopic method[47] had been already reported. But there is no analytical method for the analysis of these compounds in combination; hence an attempt was made to estimate these two drugs by RP-HPLC.

Hence primary objective of the work envisaged was to

1) Develop an analytical method for the simultaneous estimation of Loperamide hydrochloride and Norfloxacin in combination.
2) Validate the method as per ICH guidelines.
3) Determination of stability of the formulation by developed and validated assay method.

CHAPTER 4

DRUG PROFILE

- **Drug Name** : Loperamide hydrochloride [54]
- **Molecular formulae** : $C_{29}H_{33}ClN_20_2.HCl$
- **Molecular weight** : 513.5 g
- **Chemical structure**

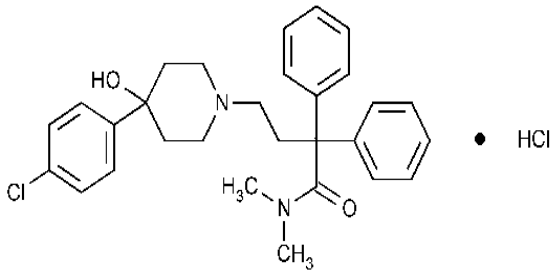

Fig 2. Structure of Loperamide hydrochloride

- **IUPAC Name** : 4-[4-(4-chlorophenyl)-4hydroxypiperidin-1-yl]-N,N-dimethyl-2,2-diphenlybutamide Hydrochloride
- **Appearance** : White or almost white powder
- **Solubility** : Slightly *soluble* in water, freely *soluble* in Alcohol and in methanol
- **Melting point** : 228˚C
- **Storage** : Protected from light
- **Loss on drying** : Not more than 0.5% determined on 1.0 g by drying in an oven at 105˚C for 4 hour
- **Sulphated ash** : Not more than 0.1% determined on 1.0 g
- **Category** : Long-acting synthetic ant diarrheal agent

- **Drug Name** : Norfloxacin [55]
- **Molecular formulae** : $C_{16}H_{18}FN_3O_3$
- **Molecular weight** : 319.3 g

Chemical structure

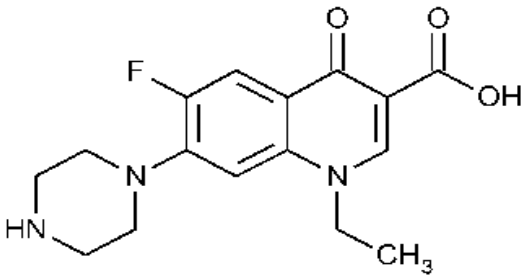

Fig 3. Structure of Norfloxacin

- **IUPAC Name** : 1-Ethyl-6-fluro-4-oxo7-(piperazine-1-yl)-1,4- Dihydroquinoline-3-carboxylic acid.
- **Appearance** : White or pale yellow, hygroscopic, photosensitive, crystalline powder**.**
- **Solubility** : Very Slightly *soluble* in water, Slightly soluble in Acetone and alcohol and in methanol
- **Melting point** : 227-228˚C
- **Storage** : In an airtight container, protected from light.
- **Loss on drying** : Not more than 1.0% determined on 1.0g by drying in an oven at 105˚C for 2 hours.
- **Sulphated ash** : Not more than 0.1% determined on 1.0 g.
- **Category** : Anti-Infective
  Anti-Bacterial Agents
  Enzyme Inhibitors
  Nucleic Acid Synthesis Inhibitors

CHAPTER 5

MATERIALS AND METHODS

6.1 Drug samples:

Norfloxacin and Loperamide HCl and are procured from Wockhardt Ltd (India) where the potency of Loperamide hydrochloride and Norfloxacin was reported to be 99.3% w/w and 99.7% w/w respectively.

6.2 Chemicals and Solvents Used:

Acetonitrile HPLC grade	–	Merck specialties
Methanol (Analytical and HPLC grade)	–	Merck specialties
Ortho phosphoric acid (Analytical grade)	–	Merck specialties
Triethylamine (Analytical grade)	_	Merck specialties
Water (HPLC grade)	–	SD fine chemicals

6.3 Instruments:

- ✓ HPLC : Waters HPLC 2695 separation module with Empower software
- ✓ UV : *PerkinElmer*
- ✓ Sonicator : Elico ultra sonicator (Sensitivity 0.0001 g)
- ✓ Weighing balance : Mettler Toledo
- ✓ pH meter : Lab India
- ✓ Solvent filtration : Millipore

6.4 METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITION:

6.4.1 Selection of wavelength:

The wavelength for the estimation of loperamide hydrochloride and norfloxacin was selected by injecting several times of mixed standards10 µg/ml each of both the drugs. The standard solution was scanned between 200-400nm. UV spectrum of both drugs shows good absorption in range between 210 – 240 nm. Hence 222 nm was selected as the detection wavelength.

6.4.2 Selection of method for separation:

Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence RP-HPLC method was selected for the initial separation because of its simplicity and suitability.

6.4.3 Selection of Chromatographic condition:

Initial Chromatographic conditions

Equipment System	:	WATERS 2695 SEPERATION MODULE
Detector	:	Photo Diode array
Injector	:	Rheodyne
Stationary phase	:	Phenomenex C18 (250×4.6 mm, 5 µm)

The optimization of HPLC parameters was done by investigating the influence of the mobile phase composition while stationary phase, detection wavelength, injection volume and column temperature were set constant.

Chromatographic conditions-1

Stationary phase	:	Phenomenex C18 (250×4.6 mm, 5 µm)
Mobile Phase	:	Water
Elution type	:	Isocratic
Solvent ratio	:	100%
Detection	:	222 nm
Flow rate	:	1 ml/min
Sample size	:	20 µL

Needle wash	:	Extended
Column temperature	:	40

Chromatographic conditions-2

Stationary phase	:	Phenomenex C18 (250×4.6 mm, 5 µm)
Mobile Phase	:	Methanol
Elution type	:	Isocratic
Solvent ratio	:	100%
Detection	:	222 nm
Flow rate	:	1 ml/min
Sample size	:	20 µL
Needle wash	:	Extended
Column temperature	:	40

Chromatographic conditions-3

Stationary phase	:	Phenomenex C18 (250×4.6 mm, 5 µm)
Mobile Phase	:	ACN
Elution type	:	Isocratic
Solvent ratio	:	100%
Detection	:	222 nm
Flow rate	:	1 ml/min
Sample size	:	20 µL
Needle wash	:	Extended
Column temperature	:	40

Chromatographic conditions-4

Stationary phase	:	Phenomenex C18 (250×4.6 mm, 5 µm)
Mobile Phase	:	ACN: Water
Elution type	:	Isocratic
Solvent ratio	:	1:1
Detection	:	222 nm
Flow rate	:	1 ml/min
Sample size	:	20 µL
Needle wash	:	Extended
Column temperature	:	40

Chromatographic conditions-5

Stationary phase	:	Phenomenex C18 (250×4.6 mm, 5 µm)
Mobile Phase	:	ACN: Water: H_3PO_4 pH 4.5 (adjusted with triethylamine)
Elution type	:	Isocratic
Solvent ratio	:	50:50:0.2 v/v/v
Detection	:	222 nm
Flow rate	:	1 ml/min
Sample size	:	20 µL
Needle wash	:	Extended
Column temperature	:	40

At the initial chromatographic condition the developing method with different mobile phases was used (water, methanol, ACN, ACN: Water) but no favorable results were obtained. But the mobile phase consists of ACN: water: phosphoric acid ph 4.5(adjusted with triethylamine) (50:40:10) give acceptable peak. Therefore for the present study ACN: water: phosphoric acid ph 4.5(adjusted with triethylamine was selected as the mobile phase.

6.4.4 Effect of pH

Keeping the ratio of mobile phase constant (50:50:0.2) ratio of ACN: water: phosphoric acid ph 4.5(adjusted with triethylamine) and the chromatogram was recorded at pH 3.5, 4.0, 4.5 with the flow rate of 1.0 ml/min. Peak merging and peak tailing was observed at 3.5 and 4.0 respectively. At 4.5 peaks with good resolution and symmetry was observed. Hence pH of 4.5 was selected for our study.

6.4.5 Effect of flow rate

Keeping the mobile phase ACN: water: phosphoric acid ph 4.5 (adjusted with triethylamine) the chromatogram was recorded at flow rate of 0.5, 1.0, 1.5 ml/min. But when using the flow rate of 0.5 and 1.5 ml/min no satisfactory results were found. At the flow rate of 1 ml/min the peaks were sharp with good resolution. Therefore for the present study 1 ml/min was selected as the flow rate.

6.4.6 Fixed chromatographic condition

Stationary phase	:	Phenomenex C18 (250×4.6 mm, 5µm)
Mobile Phase	:	ACN: Water: H_3PO_4
		pH 4.5 (adjusted with triethylamine)
Elution type	:	Isocratic
Solvent ratio	:	50:50:0.2 v/v/v
Detection	:	222 nm
Flow rate	:	1 ml/min
Sample size	:	20 µL
Needle wash	:	Extended
Column temperature	:	40

6.5 Preparation of Standard stock solutions:

6.5.1 Standard stock solution A:

Accurately weighed 200 mg of Norfloxacin and transferred into a 100 ml volumetric flask, dissolved in 25 ml of mobile phase, sonicated for 5 min and the final volumes make up with mobile phase.

6.5.2 Standard stock solution B:

Accurately weighed 20 mg of Loperamide hydrochloride and transferred into a 100 ml volumetric flask, dissolved in 25 ml of mobile phase, sonicated for 5 min and the final volumes made up with mobile phase.

6.5.3 Preparation of working Standard solutions:

Accurately pipette out 10 ml of standard stock solution A and 1 ml of standard stock solution B into a 100 ml volumetric flask, dissolved and make up to the final volume with mobile phase.

6.5.4 Preparation of sample solution:

Accurately weighed 210 mg of powdered sample and transferred into a 100 ml volumetric flask, dissolved with 25 ml of mobile phase, sonicated for 10 min and the final volume was made up with methanol and filtered through whatmann filter paper No: 41. The final mixed sample solution was prepared, corresponding to 200 µg/ml of norfloxacin and 2 µg/ml of Loperamide hydrochloride with the same solvent.

6.6 HPLC Method Validation:

After developing a method its validation is necessary to prove the suitability of the method for the intended purpose. The developed HPLC method was validated for accuracy, precision, linearity, robustness, limit of detection (LOD), limit of quantification (LOQ) and system suitability. The validation was carried out as per ICH guidelines (1996). The procedures followed for the validation of the method are given below.

6.6.1 System suitability:

System suitability determination was carried out by replicate analysis of the system suitability solution of both drugs. It was performed by calculating the chromatographic parameters like column efficiency, resolution, peak asymmetry factor etc.

6.6.2Specificity:

Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. The specificity of the method was determined by complete separation of Loperamide and Norfloxacin with parameters like retention time (Rt), resolution (Rs) and tailing factor (T), peak purity. Peak purity of the method was evaluated by calculating the difference between peak angle (θ_p) and peak threshold angle (θ_{th}). If $\theta_p < \theta_{th}$, the peak is considered spectrally homogeneous; otherwise the peak is influenced by the presence (i.e., additional absorption) of another substance. Peak purity evaluation was performed with the objective to prove that the method is specific in nature.

6.6.3 Linearity and Range (Calibration curve):

A calibration curve was plotted over a concentration range of (5-25 µg/ml) for both the drugs. The chromatogram of each solution was recorded at 222 nm. Calibration curve were constructed for LOP and NOR by plotting peak area versus concentration. Each reading was the average of five replicate injections. The linearity was assessed by calculating the slope, y-intercept and coefficient of determination (R^2).

6.6.4 Precision:

The precision of an analytical method is usually expressed as the standard deviation, relative standard deviation or coefficient of variation of variance of a series of measurement. It was examined by performing the intra-day and inter-day studies. Three replicate injections of the mixture of standard solution at five concentration levels (5, 10, 15 µg/ml) were carried out and the standard deviation and relative standard deviation was calculated. The intra-day studies was performed at intervals of 3h in a day, while the inter day studies was performed over in alternative days.

6.6.5 Accuracy:

Determination of accuracy was done by calculating the recoveries by using standard addition method. Known amount of the standards (50, 100, 150 %) were added into the pre-analyzed sample solutions and the amount of these standard were estimated by using assay method.

6.6.6 Robustness:

The robustness of the method is to measure the ability of the method to remain unaffected by small variations in parameters of the method and there by provides an indication of its reliability during normal usage. The robustness of the proposed method was evaluated by changing the flow rate, column and pH.

6.6.7 Ruggedness:

It expresses the precision within laboratory variations like different days, different analyst, and different equipments. Ruggedness of the method was assessed by spiking the standard six times in two different days with different analyst.

6.6.8 Limit of Detection (LOD):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD of both the analytes were determined by k SD/b where k is a constant (3.3 for LOD), SD is standard deviation of the analytical signal, and b is the slope of the concentration/response graph.

6.6.9 Limit of Quantification (LOQ):

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low level of compounds in sample matrices and is used particularly for the determination of impurities or degradation products. LOQ of both the analytes were determined by *k* SD/*b* where *k* is a constant (10 for LOQ), SD is the standard deviation of the analytical signal, and *b* is the slope of the concentration/response graph.

6.7 Accelerated stability study:

The accelerated stability studies are done for the verification of quality of the drug under the influence of various environmental factors. The stability of the formulation was analyzed by performing the assay of the formulation by the developed RP-HPLC method. The samples are stored at 40^0/70% and they were analyzed at 1, 2, 3 and 6 months.

CHAPTER 6

RESULTS

Table 1. System suitability parameters

Parameters	NOR	LOP
Number of theoretical plates	80121	7543
Asymmetry factor	0.97	1.09
Resolution	2.44	
Limit of detection	1.17	0.16
Limit of quantification	3.59	0.39
Linearity range	5-25 µg/ml	5-25 µg/ml

Table 2. Linearity and range (n=5)

S.NO.	Conc. of NOR (µg/ml)	Mean Peak area of NOR
1	5	164936
2	10	321621
3	15	471238
4	20	658712
5	25	814816

Table 3. Linearity and range (n=5)

S.NO.	Conc. of LOP (μg/ml)	Mean Peak area of LOP
1	5	122618
2	10	239261
3	15	370322
4	20	494158
5	25	596517

Table 4. Recovery data for the proposed method

Drug	Quantity added %	Quantity found, mg/ml	Recovery, %	%RSD
NOR	50	99.48	99.46	0.84
	100	200.02	100.13	0.69
	150	299.97	99.85	1.26
LOP	50	0.97	99.94	0.64
	100	2.01	100.05	0.77
	150	3.19	100.76	0.41

Table 5. Intraday assay precision data (n=3)

Concentration (µg/ml)	Intraday			
	Peak area		Peak area	
	Mean	%RSD	Mean	%RSD*
	NOR		LOP	
5 µg/ml	165494	1.106	121817	0.572
10 µg/ml	322867	0.338	242747	0.474
15 µg/ml	487606	0.319	368093	0.277

Table 6. Interday assay precision data (n=3)

Day	Concentration	Interday			
		Peak area		Peak area	
		Mean area	%RSD	Mean	%RSD*
		NOR		LOP	
Day 1	5 µg/ml	165494	1.106	121817	0.572
	10 µg/ml	322867	0.338	2427467	0.474
	15 µg/ml	487606	0.319	368093	0.277
Day2	5 µg/ml	163972	0.817	120964	0.621
	10 µg/ml	323452	0.512	241961	0.371
	15 µg/ml	489213	0.456	367919	0.466
Day3	5 µg/ml	164764	0.931	122176	0.361
	10 µg/ml	326109	0.792	241635	0.217
	15 µg/ml	483946	0.521	370264	0.839

Table 7. Robustness testing of the method

Parameters changed		% Assay	
		NOR	**LOP**
Column brand	Phenomenex	99.66	99.29
	Hypersil	101.12	98.63
	Waters	98.69	99.82
pH	4.3	98.88	99.17
	4.5	99.39	100.39
	4.7	98.18	98.85
Flow rate	0.9 ml/min	99.29	99.37
	1.0 ml/min	100.93	98.49
	1.1 ml/min	101.53	98.24

Table 8. Analysis of formulation

Components	**Label claim mg/g**	**Amount found mg/g**	**% claim**
NOR	200	199.93	99.88
LOP	2	2.05	100.14

Table 9. Ruggedness testing of the method

Drug		Concentration (µg/ml)	Mean peak area	% RSD
NOR	Day 1 Analyst 1	10	321621	0.348
	Day 2 Analyst 2	10	322119	0.721
LOP	Day 1 Analyst 1	10	239261	0.624
	Day 2 Analyst 2	10	241174	0.297

Table 10. Accelerated stability study

	DRUGS	0 month	1 month	2 month	3 month	6 month
ASSAY	NOR	99.8%	99.4%	99.1%	98.7%	98.1%
	LOP	100.1%	99.7%	99.3%	99.1%	98.5%

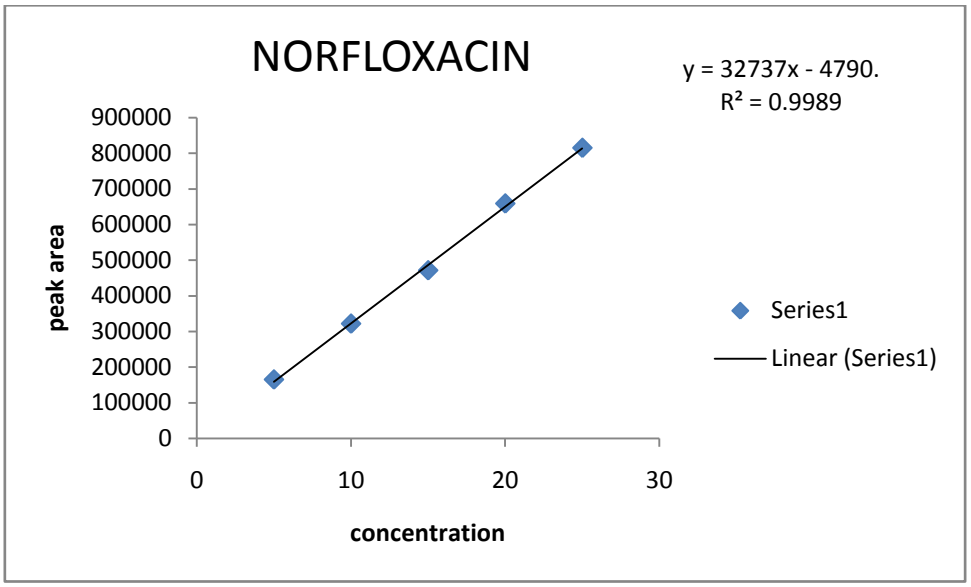

Fig 4. Calibration curve of Norfloxacin.

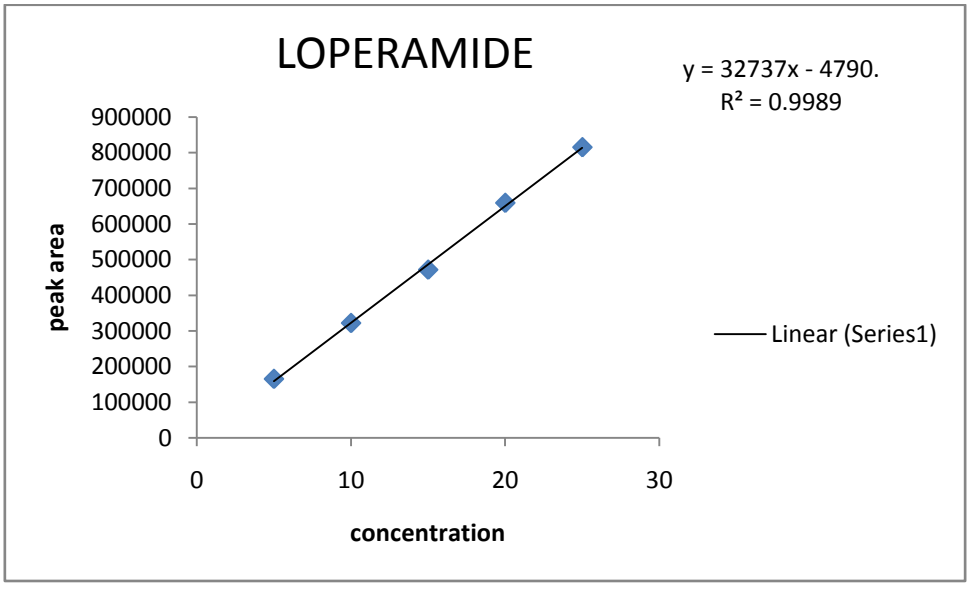

Fig 5. Calibration curve of Loperamide.

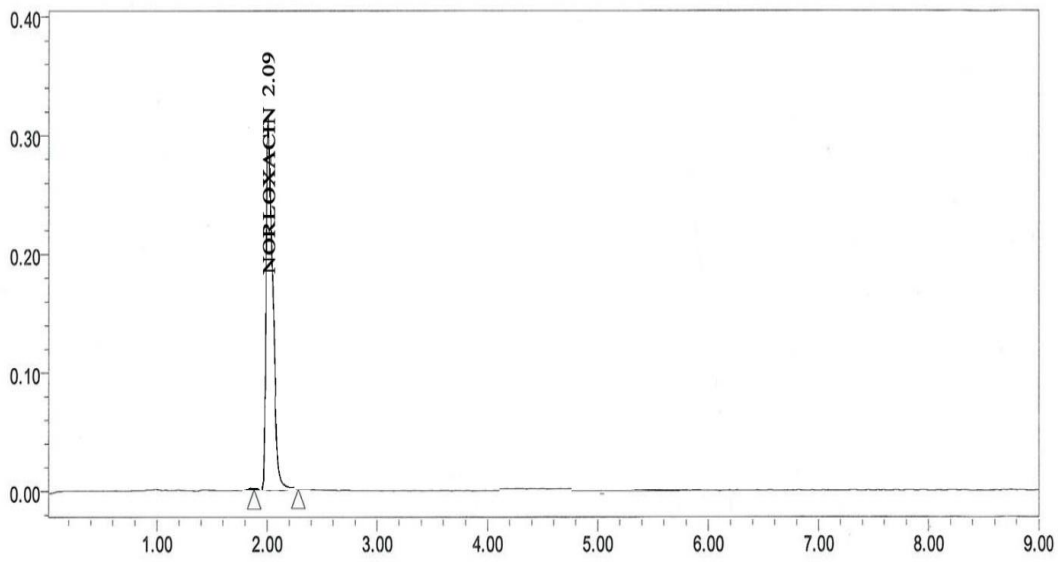

Fig 6. Chromatogram for retention time of norfloxacin

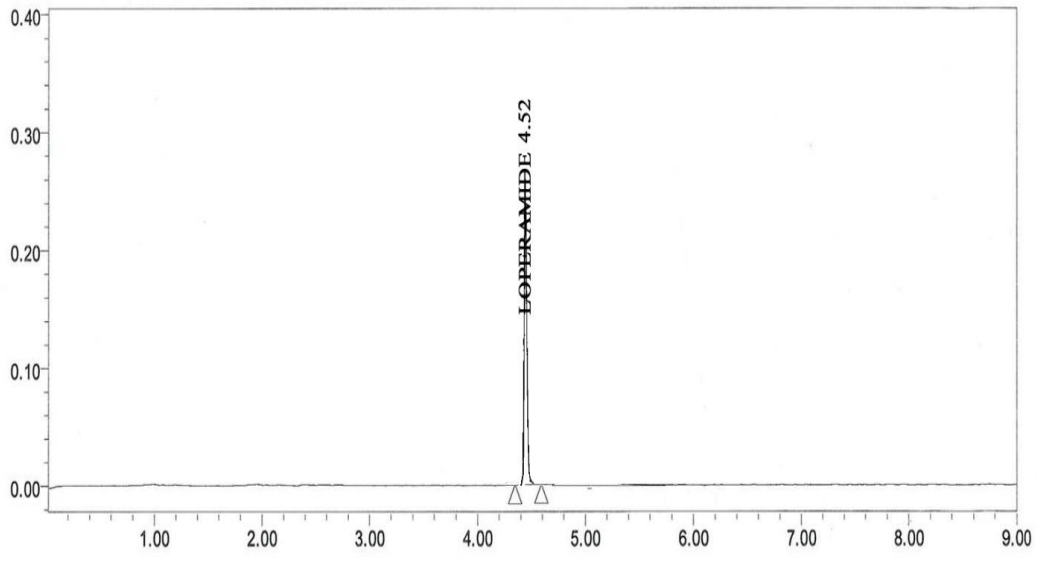

Fig 7.Chromatogram for retention time of loperamide

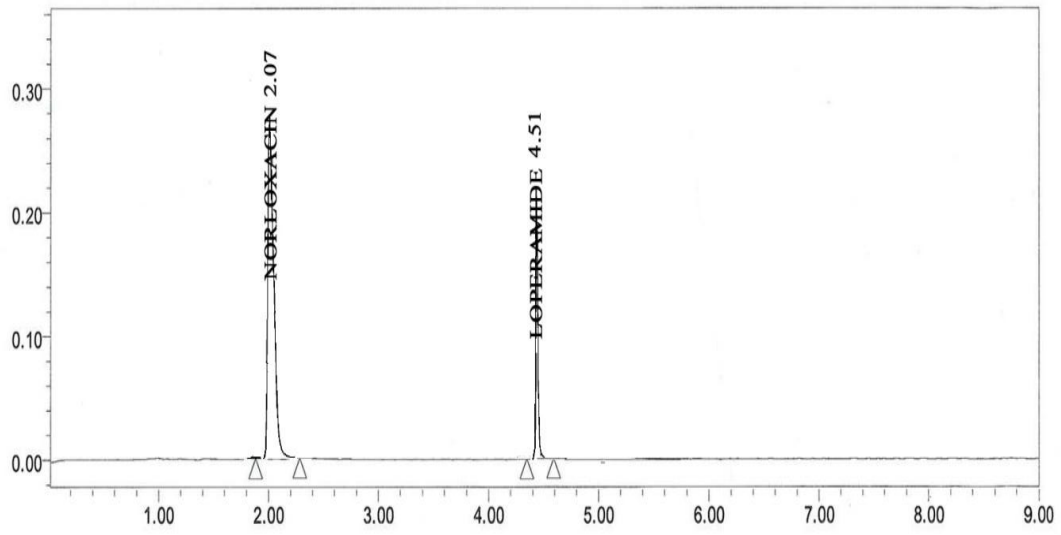

Fig 8. Chromatogram of Linearity Standard solution of NOR and LOP 5 µg/ml

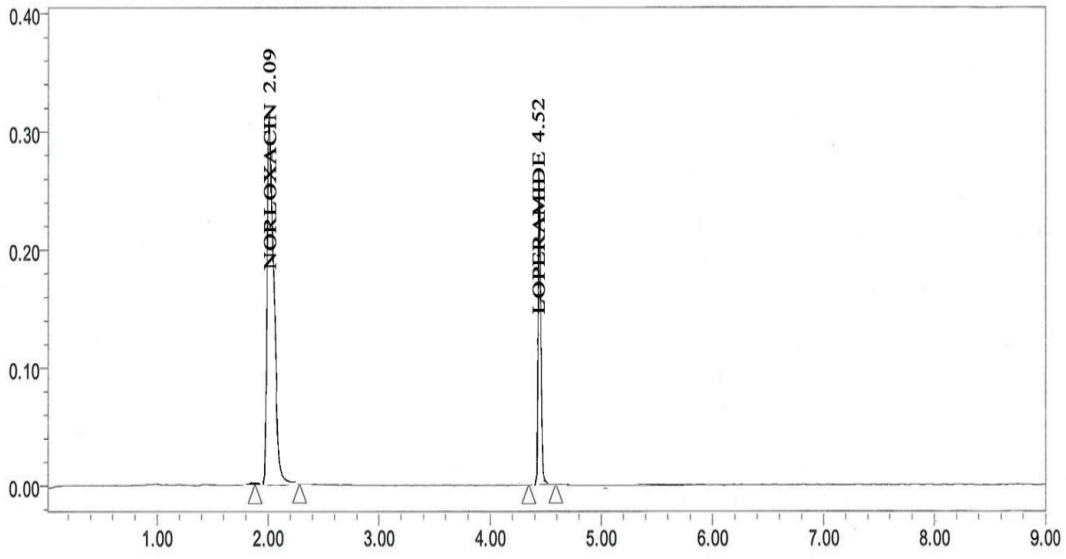

Fig 9. Chromatogram of Linearity Standard solution of NOR and LOP 10 µg/ml

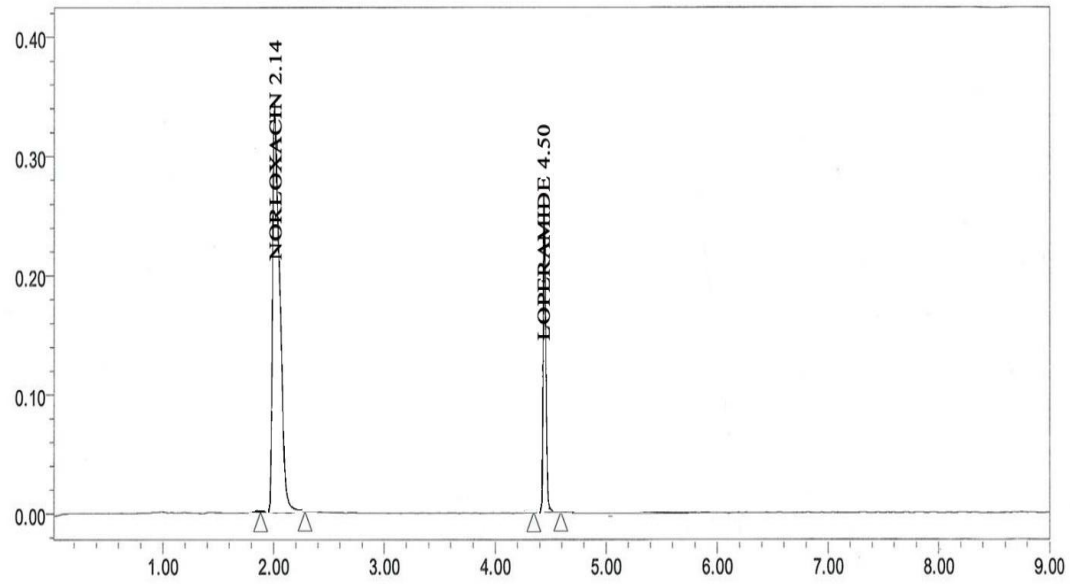

Fig 10. Chromatogram of Linearity Standard solution of NOR and LOP 15 µg/ml

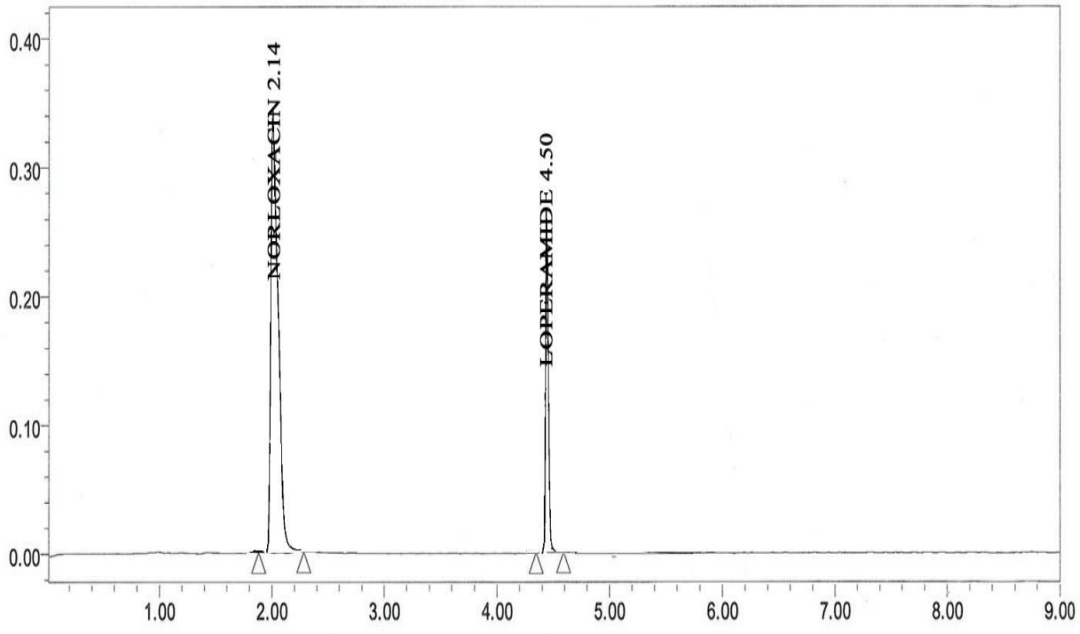

Fig 11. Chromatogram of Linearity Standard solution of NOR and LOP 20 µg/ml

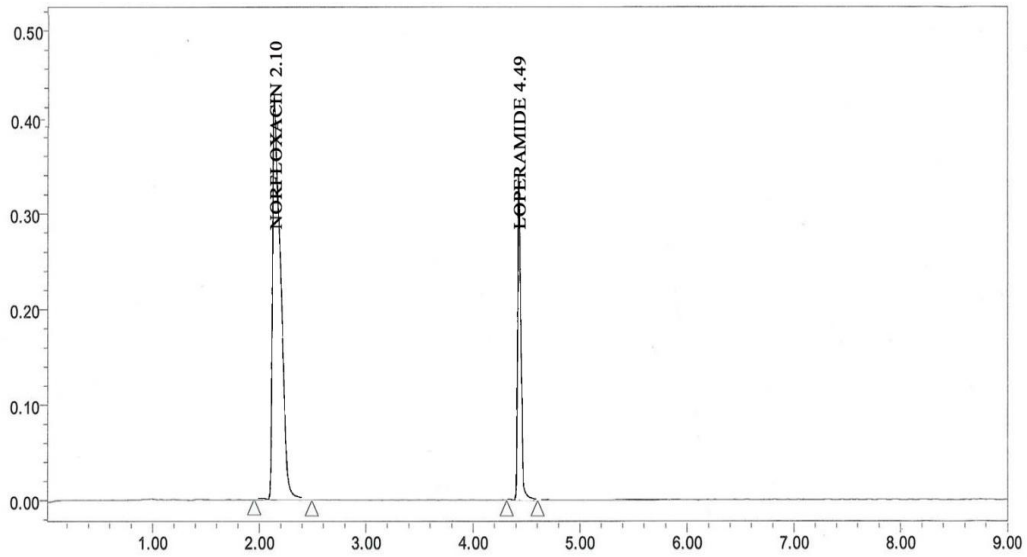

Fig 12. Chromatogram of Linearity Standard solution of NOR and LOP 25 µg/ml

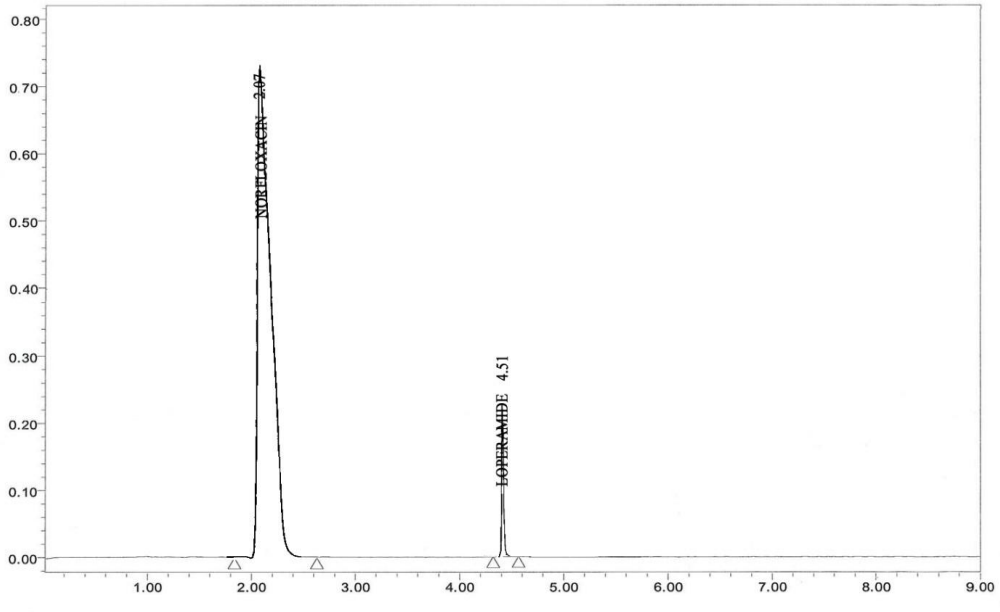

Fig 13. Chromatogram of sample solution of NOR and LOP

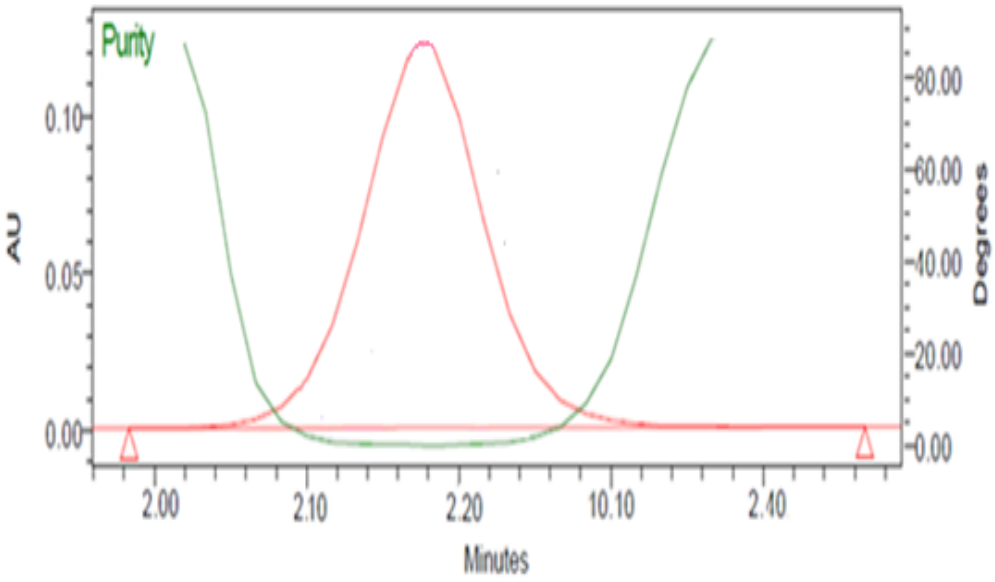

Fig 14. Chromatogram of sample Peak profile of Norfloxacin

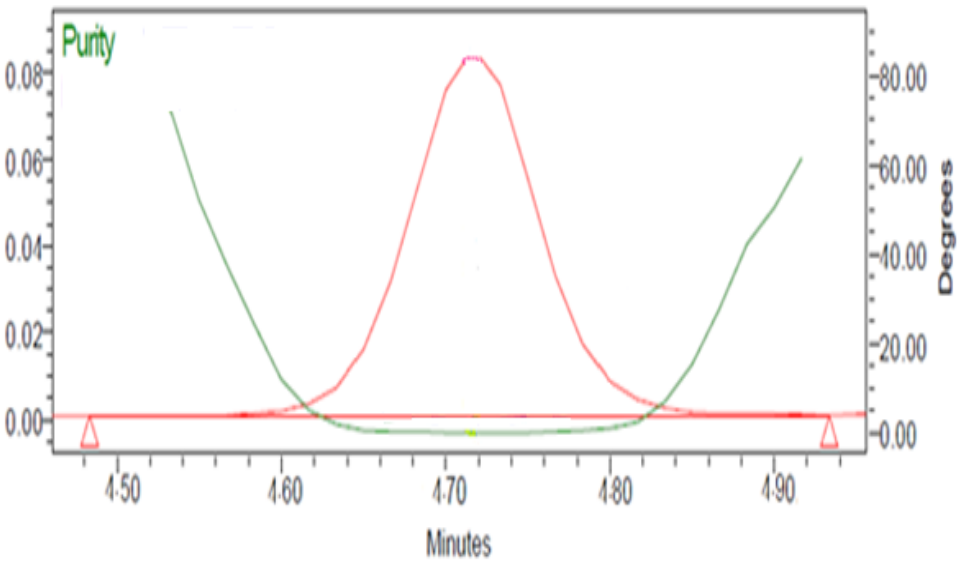

Fig 15. Chromatogram of sample Peak profile of Loperamide

CHAPTER 7
DISCUSSION

Combination of Loperamide and Norfloxacin is used as an anti-diarrheal agent. From the literature survey it was found that no method has been reported for the simultaneous estimation of LOP and NOR in combination. Hence a validated RP-HPLC method has been developed for the simultaneous estimation of these drugs in combined form.

Optimization of chromatographic conditions

Suitable selection of the method depends upon the nature of the sample, its molecular weight and solubility. Hence the drugs selected in the present study are polar in nature, RP-HPLC method was selected for the initial separation because of its simplicity and suitability. The method was performed on a Phenomenex C18 (250×4.6 mm, 5 µm) column.

For the determination of wavelength for the simultaneous estimation of LOP and NOR an UV overlay spectrum was recorded in the range of 200-400 nm and both drugs shows a good absorption in the range between 210 and 230 nm. Hence 222 nm was selected as wavelength of detection.

The optimization of HPLC parameters was done by investigating the influence of the mobile phase composition while stationary phase, detection wavelength, injection volume and column temperature were set constant.

In the case of 1st chromatographic Conditions water was selected as mobile phase at a ratio of 100% but resulted in poor elution of peaks. Hence methanol was selected instead of water at a ratio of 100% and chromatograms were recorded in Conditions 2. While using methanol as the mobile phase, peak merging was observed. As a result ACN was selected as mobile phase at a ratio of 100% in condition 3.In this peak resolution was obtained but the peak symmetry was not good and not within the acceptance criteria. Therefore, ACN: Water: H_3PO_4 was selected for the condition 4 as mobile phase and peaks with poor resolution were obtained. So in condition 5 the pH of the above mobile phase was adjusted to 4.5 with triethylamine. Finally in the

chromatographic condition 5 the peaks with good resolution and peak symmetry were observed and therefore this was selected as the mobile phase system for the determination of the drugs.

Method validation

The method developed for the determination of loperamide hydrochloride and norfloxacin in combined dosage form was validated for accuracy, precision, linearity, robustness, ruggedness, system suitability, LOD and LOQ. The validation was carried out as per ICH guidelines.

System suitability

Five duplicate injections of the system suitability solution gave %RSD value for peak area within 2%, indicating low difference of the calculated values (Table 1). The symmetry of all peaks was < 2. The resolution (R) between Loperamide and Norfloxacin was 5.11, representing a high degree of peak separation (R>2). The efficiency of the column, as uttered by the number of theoretical plates, was more than 2000. These results revealed the suitability of the HPLC system and conditions of the developed method.

Specificity

Specificity determination was done by analyzing loperamide hydrochloride and norfloxacin simultaneously. Peaks for the drugs were observed to be well separated and not interfered with any other components. Peak threshold angle (θ_{th}) of all the peaks was found to be larger than that of peak angle (θ_p), values are shown in Figures (h,i) indicating the purity of the peaks.

Calibration curve (Linearity)

Five working solutions each for loperamide and norfloxacin in the range of 5-25 µg/ml were prepared respectively. Multiplicative of each solutions were injected and the linear regressions analysis of NOR and LOP were constructed by plotting the peak area of the analytes (*y*) versus analyte concentration (µg /ml) on (*x*) axis**.** The slopes, *y*-intercepts and correlation coefficients (R^2) obtained from regression analysis are shown in (Table 2, 3, Figure 11, 12). The regression equation were $y = 32737x - 4790$ ($R^2 = 0.9989$), $y = 24054x + 3766$ ($R^2 = 0.9985$) for NOR and LOP respectively. Linear regression analysis revealed that the R^2 values for both the drugs were >0.999

confirming the linear relationship between the concentration of the analytes and area under the curve.

Precision

The intra-day precision was evaluated by analyzing five replicate samples of each at three different level (5, 10, and 15) containing pure drug. The % RSD value revealed high precision of the developed method. The inter-day precision was evaluated by analyzing five replicate samples of each at three different level (5, 10, and 15) containing pure drug. The % RSD value revealed high precision of the developed method.

Accuracy

The recovery of the investigated drugs ranged from 99.46-100.76%, and their %RSD values were all < 2% as shown in Table 4. It was identified from recovery tests that all percentage recoveries were within 99-101%, signifying the excellent accuracy of the developed method.

Robustness

As shown in Table 7 robustness data of the mean obtained (n=6) for each factor studied, including that the selected factors remained unaltered by small variations of these parameters. The individual recovery was obtained and the mean were between 98-102% for both loperamide and norfloxacin. Thus the method was found to be robust for change in column manufacturer, pH and flow rate.

Ruggedness

According to ICH guidelines ruggedness was performed during the development of analytical procedure. By comparing inter-day and intraday precision result done by two analyst the ruggedness of the method can be assessed. The % RSD values were found to be less than 2 for intra-day and inter-day assay of Norfloxacin and Loperamide indicates the ruggedness of the method as shown in table 9.

Limit of detection (LOQ) and Limit of quantification (LOD)

The detection limit was found to be 1.17 for Norfloxacin, 0.16 µg/ml for Loperamide and the quantification limit was found to be 3.59 µg/ml for Norfloxacin and 0.39 µg/ml for Loperamide. These results show that the method gives enough sensitivity.

Accelerated stability studies

The content of NOR and LOP evaluated for the fabricated formulation after accelerated stability study at 40^0C/75%RH for a period of 6 months. Assay values were found to be satisfactory (±10%) as compared to the label claim of the drugs as shown in Table 10.

CHAPTER 8

SUMMARY AND CONCLUSION

SUMMARY

- For the quantitative estimation of Norfloxacin and Loperamide HCl in combined capsule dosage form a RP-HPLC method was developed
- The developed method was validated for different parameters like system suitability, specificity, linearity, accuracy, precision, LOD, LOQ, robustness and ruggedness. All these studies was carried out as per ICH guidelines
- Form the developed method the retention time was found to be 2.09 and 4.51 min for NOR and LOP respectively.
- The developed method was found to be linear in the range of 5-25 µg/ml for both NOR and LOP.
- The detection and quantification limits were found to be 1.17 and 3.59 µg/ml for NOR and 0.16 and 0.39 µg/ml for LOP respectively.
- The recovery of the components ranged from and their %RSD values were found to be within the limit (< 2%). The precision result also revealed that the % RSD values of both NOR and LOP were less than 2%.
- It was observed that the developed and validated method for the estimation of NOR and LOP was found to be robust and rugged.
- The developed and validated method was successfully applied for the quantitative and qualitative estimation of NOR and LOP in combined capsule formulation.
- The/ formulation containing NOR AND LOP was found to be stable after accelerated stability study performed at 40^0c/75%RH for a period of 6 months.

CONCLUSION

The developed validated RP-HPLC method was successfully applied for the routine simultaneous estimation of NOR and LOP in capsule dosage form and can be applicable for its stability studies. The method was cost effective due to low retention time (less than 5 min for both drugs) and less consumption of mobile phase. No interferences of excipients were observed in the sample chromatogram. The developed method can be applicable for the routine analysis of these drugs in pharmaceutical formulation.

CHAPTER 9

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