"DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC AND RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN AND LOTEPREDNOL IN THEIR COMBINED PHARMACEUTICAL DOSAGE FORM"

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IN

PHARMACEUTICAL ANALYSIS

BY

RAVISINH VIKRAMSINH SOLANKI, B.PHARM
(11MPH307)

UNDER THE GUIDANCE OF

MR. NRUPESH R. PATEL

DEPARTMENT OF PHARMACEUTICAL ANALYSIS
INSTITUTE OF PHARMACY
NIRMA UNIVERSITY
AHMEDABAD-382481
GUJARAT, INDIA

CERTIFICATE

This is to certify that ***Mr. Ravisinh V. Solanki*** *has prepared his thesis entitled* ***"DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC AND RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN AND LOTEPREDNOL IN THEIR COMBINED PHARMACEUTICAL DOSAGE FORM"****, in partial fulfillment for the award of M. Pharm. degree of the Nirma University, under my guidance. He has carried out the work at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University.*

Guide:

NRPatel

Mr. Nrupesh R. Patel,
M. Pharm.,
Assistant Professor,
Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University.

Forwarded Through:

Dr. Priti J. Mehta
M. Pharm., Ph.D
Professor & Head,
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University

Dr. Manjunath Ghate
M. Pharm., Ph.D.
Director
Institute of Pharmacy,
Nirma University.

Date: 15th May, 2013

DECLARATION

*I declare that the thesis "**DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC AND RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN AND LOTEPREDNOL IN THEIR COMBINED PHARMACEUTICAL DOSAGE FORM" has been prepared by me under the guidance of Mr. Nrupesh R. Patel, Professor, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.*

Mr. Ravisinh V. Solanki
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University,
Sarkhej - Gandhinagar Highway,
Ahmedabad-382481,
Gujarat, India.

Date: 15th May, 2013.

DEDICATED

TO

MY BELOVED

FAMILY

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Date: 15th May, 2013
Mr. Ravisinh V. Solanki
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University.

LIST OF ABBREVIATIONS

ABBREVIATION	FULL FORM
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
GLC	Gas Liquid chromatography
GC	Gas chromatography
GR	glucocorticoid receptor
IOP	Intra Ocular pressure
IUPAC	International Union of Pure and Applied Chemistry
CAS No.	Chemical Abstract Service Number
IP	Indian Pharmacopoeia
JP	Japanese Pharmacopoeia
BP	British Pharmacopoeia
API	Active Pharmaceutical Ingredient
°C	Degree centigrade
±	Plus or Minus
AR	Analytical Reagent
pKa	Dissociation constant
T_{max}	Time at which maximum concentration attained
C_{max}	Maximum Plasma Concentration
USP	United states of pharmacopoeia
LE	Loteprednol etabonate
$T_{1/2}$	T Half time
h	Hour
<	Less than
λ	Lambda
%	Percentage
Conc.	Concentration
AUC	Area Under Curve
Mg	Microgram
µL	Microliter
Abs.	Absorbance

ACN	Acetonitrile
cm	Centimeter
FDA	Food and Drug Administration
Fig.	Figure
FT-IR	Fourier Transform Infrared spectrometry
g	Gram
i.d.	Internal Diameter
IS	Internal Standard
ICH	International Conference on Harmonization
L	Liter
L/h	Liter per hour
LC	Liquid Chromatography
MeOH	Methanol
mg	Milligram
Min	Minimum
mM	Milimolar
LLOQ	Lower Limit of Quantification
LOQ	Limit of Quantification
min	Minute(s)
mL	Mililiter
mm	Milimeter
MS	Mass Spectrometry
mPa	MilliPascal
n	Number
No.	Number
ng	Nanogram
Pg.No.	Page number
pKa	Partition coefficient
Ppb	Part Per Billion
Ppm	Part Per Million
Ref.No.	Reference number
Rf	Retention Factor
RP	Reverse Phase

R.S.D.	Relative Standard Deviation
Rt	Retention Time
S.D.	Standard Deviation
Sec.	Second
Sol^n	Solution
Sr.No.	Serial number
Std.	Standard
M	Meter
Max	Maximum
TLC	Thin Layer Chromatography
UV	Ultra violate
UV/Vis	Ultra violate/Visible
Vol.	Volume
v/v	Volume/Volume
w/v	Weight / Volume

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A simple, sensitive, rapid, accurate and precise absorption ratio method has been developed for concurrent estimation of Loteprednol and Levofloxacin in combined dosage form. Ratio of absorbance at two selected wavelengths was calculated. First wavelength is absorption maxima of respective drug and second wavelength is isoabsorptive point at which both drugs give same absorbance. Levofloxacin shows absorbance maxima at 298.5 nm and Loteprednol shows absorbance maxima at 237.5 nm in methanol. The isoabsorptive point of Levofloxacin and Loteprednol was found to be at 269.29 nm. Linearity was constructed in the concentration range of 5-25 µg/ml. Promising values of correlation coefficient for Loteprednol (R^2=0.9984) and Levofloxacin (R^2=0.9990) proves that method is linear. Furthermore, the method was successfully validated in terms of various validation parameters as suggested by ICH guidelines. The developed method was successfully applied for estimation of both Loteprednol and Levofloxacin from its synthetic mixture.

A simple, specific and accurate high performance liquid chromatography (HPLC) method was also developed and validated for simultaneous quantification of Levofloxacin and Loteprednol etabonate in their combined dosage form. Chromatography was performed using C_8 column (Zorbax) 250 mm ×4.6 mm i.d., 5µm with Acetonitrile : ammonium acetate buffer (20 mM) added 0.5 mL TEA/500 ML adjusted to pH-3.1 by Glacial acetic acid, as mobile phase. Wavelength used was 269 nm. The gradient system was applied to separate both the drugs. Linearity was constructed in concentration range of 75 to 375 µg/ml for Levofloxacin and 25 to 125 µg/ml for Loteprednol etabonate. Promising values of correlation coefficient for Loteprednol (R^2=0.997) and Levofloxacin (R^2=0.999) proves that method is linear. Furthermore, the method was successfully validated in terms of various validation parameters as suggested by ICH guidelines. The developed method was successfully applied for estimation of both Loteprednol and Levofloxacin from its synthetic mixture.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO MULTICOMPONENT FORMULATION [1]

Market is flooded with combination of drugs in various dosage forms. The multi-component formulations have gained a lot of importance nowadays due to greater patient acceptability, increased potency, multiple action, fewer side effects and quicker relief.

The multi-drug therapy is an ancient phenomenon to combat interrelated symptoms of diseased status of human beings. Since it ensure timely and complete medication for disorder and it has patient compliance, as it reduces the number of formulations to be taken at a time. Therefore, the pharmaceutical formulations with combinations of drugs have shown an increasing trend to counteract other symptoms specific to one drug and formulation, and hence analytical chemist will have to accept the challenge of developing reliable methods for analysis of drugs in such formulation.

Simultaneous analysis procedures are now being used more frequently for estimation of drugs in multi-component pharmaceutical formulations due to their inherent advantages viz. avoid time consuming extraction and separation, economical in the sense that use of expensive regents is minimized are equally accurate and precise. For the estimation of multi-component formulation, the instrumental techniques, which are commonly employed, are spectrophotometry, Gas Liquid chromatography, high performance thin layer chromatography (HPTLC), HPLC etc.

Spectrophotometric multi-component analysis:

Absorption spectroscopy is one of the most useful and widely used tools available to the analyte for quantitative analysis. The relation between the concentration of analyte and the amount of light absorbed is the basis of most analytical applications of molecular spectroscopy. This method of analysis is gaining importance due to simple, rapid, precise, highly accurate and less time consuming. Spectrophotometric multi-component analysis can be applied where the spectra of drugs overlaps. In such cases of overlapping spectra, simultaneous equation can be framed to obtain the concentration of individual component;

otherwise multi-component analysis can be applied on any degree of spectral overlap provided that two or more spectra are not similar exactly.

High performance liquid chromatography (HPLC):

This technique is based on the same method of separation as classical column chromatography i.e. adsorption, partition, ion exchange and gel permeation but it differ from column chromatography, in that mobile phase is pumped through the packed column under high pressure. The technique is most widely used for all the analytical separation technique due to its sensitivity, its ready adaptability to accumulate quantitative determinations, its suitability for separating nonvolatile species or thermally fragile ones. In normal HPLC, polar solids such as silica gel; alumina (Al_2O_3) or porous glass beads and nonpolar mobile phase such as heptane, octane or chloroform are used but if the opposite case holds, it is called as reversed phase HPLC.

High performance thin layer chromatography (HPTLC):

The principle is based on plane chromatography. The mobile phase normally is driven by capillary action. The prominent advantages of this technique includes possibilities of separating of up to 70 samples and standard simultaneously on a single plate leading to high throughout, low cost analogs and the ability to construct calibration curves from standard chromatography under the same condition as the sample. Analyzing a sample by use of multiple separation steps and static post chromatographic detection procedures with various universal and specific visualization regents that are possible because all the sample components are stored on the layer without the chance of loss.

Gas chromatography (GC):

GC is one of the most extensively used separation technique in which separation is accomplished by partitioning solute between a mobile gas phase and stationary phase, either liquid or solid. The chief requirement is same degrees of stability at the temperature necessary to maintain the substance in gas state.

1.2 INTRODUCTION TO DISEASE

The eye is vulnerable to damage from relatively low levels of intraocular inflammation. The blood-aqueous and blood-retinal barriers usually limit penetration of protein and cells from the peripheral circulation, while regulatory molecules and cells in the eye actively suppress immunologic responses [2]. Nevertheless, ocular inflammatory conditions and surgical trauma induce changes in the blood-aqueous and blood-retinal barriers [2–4]. As a result, immune cells and mediators of inflammation enter the eye, resulting in the classical clinical signs and symptoms of ocular inflammation including redness, pain, swelling, and itching [5]. Ocular inflammation, if left untreated, may lead to temporary or permanent loss of vision [6].

Topical corticosteroids are useful for the management of anterior segment inflammation. Corticosteroids elicit numerous potent anti-inflammatory effects [7]. For instance, they suppress cellular infiltration, capillary dilation, the proliferation of fibroblasts, collagen deposition, and eventually scar formation; they stabilize intracellular and extracellular membranes; and they increase the synthesis of lipocortins that block phospholipase A_2 and inhibit histamine synthesis in the mast cells. Inhibition of phospholipase A_2 prevents the conversion of phospholipids to arachidonic acid, a critical step in the inflammatory cascade. Corticosteroids also increase the enzyme histaminase and modulate transcription factors present in mast cell nuclei.

Corticosteroids mediate their anti-inflammatory effects primarily through the modulation of the cytosolic glucocorticoid receptor (GR) at the genomic level [8, 9]. After corticosteroids bind to the GR in the cytoplasm, the activated corticosteroid-GR complex migrates to the nucleus, where it upregulates the expression of anti-inflammatory proteins and represses the expression of proinflammatory proteins. However, recent work suggests that the activated corticosteroid-GR complex also elicits nongenomic effects, particularly the inhibition of vasodilation, vascular permeability, and migration of leukocytes [8,10]. In addition, corticosteroids mediate anti-inflammatory activity through membrane-bound GR-mediated nongenomic effects and through direct nonspecific interactions with cellular membranes [10, 11].

Because the GR is involved in a plethora of signalling pathways; in fact, more than 5000 genes are expressed or suppressed following glucocorticoid exposure [12] long-term use or high dosages of corticosteroids can result in adverse drug reactions (ADRs) such as increased Intra ocular pressure (IOP) [13, 14]. Most studies implicate the involvement of trabecular meshwork (TM) cells and myocilin gene expression in the mechanism of corticosteroid-induced IOP increase. Steroids decrease the outflow of aqueous humor by inhibiting the degradation and/or enhancing the deposition of extracellular matrix material within the TM and/or cross-linking of actin fibres between TM cells [15]. Structural changes in the TM, in turn, result in corticosteroid-induced ocular hypertension, which can progress to secondary iatrogenic open-angle glaucoma [16]. Myocilin, initially referred to as TM-inducible glucocorticoid response or TIGR gene product, is a 55-kDa protein induced after exposure of TM cells to dexamethasone for 2-3 weeks, which is also closely associated with decreased aqueous humor outflow and steroid-induced IOP increase [17]. Different mutations within the myocilin gene lead to a variety of glaucoma phenotypes in both juvenile and adult-onset primary open-angle glaucoma, providing further evidence for its role in steroid-induced IOP [15].

Another ADR associated with corticosteroid use is the formation of cataract. However, the mechanism of steroid-induced cataract formation appears to be chemically based and not likely to be related to the downstream effects of GR activation. Currently, the most prominent hypothesis for cataract formation involves nonenzymatic formation of Schiff base intermediates between the steroid C-20 ketone group and nucleophilic groups such as ε-amino groups of lysine residues of lens proteins [18]. The formation of Schiff bases is followed by a Heyns rearrangement of the adjacent C-21 hydroxyl group, resulting in stable anime-substituted adducts (Figure 1) [18]. While this covalent binding mechanism could account for cataract formation with C-20 ketone-based corticosteroids, other mechanisms of steroid-induced cataract formation may exist. Interestingly, covalent adducts have been observed only in steroid-induced cataract, not in other cataracts.

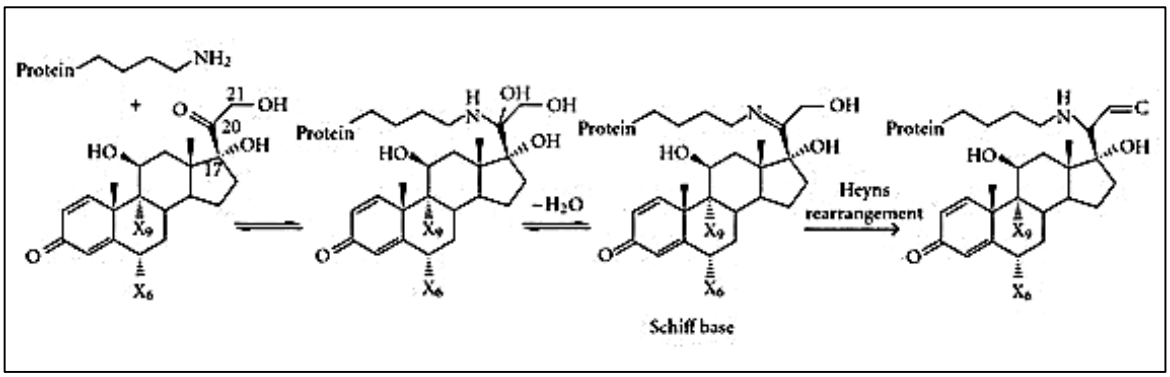

Figure 1.1: Mechanism of steroid-induced cataract formation adapted from [18].

1.3 RATIONALE OF DRUG COMBINATION [19-21]

Levofloxacin, one of the commonly used fluoroquinolone antimicrobials, is the active S-isomer isolated from the racemic Ofloxacin. Its antibacterial action is twice as active as the racemate Ofloxacin *in vitro*. Levofloxacin possesses a broad spectrum of activity against various bacteria, including gram-positive and gram-negative microorganisms [19]. It is also active against causes of atypical respiratory infection such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* [20]. Because of its excellent antibacterial activity and low frequency of adverse effects on oral administration, levofloxacin has been widely used for the treatment of infectious diseases of eye.

Loteprednol etabonate (LE) is a "soft" steroid belonging to a unique class of glucocorticoids. Loteprednol etabonate is structurally similar to other glucocorticoids. However, the number 20 position ketone group is absent. It is highly lipid soluble which enhances its penetration into cells. Loteprednol etabonate is synthesized through structural modifications of prednisolone- related compounds so that it will undergo a predictable transformation to an inactive metabolite. It first binds to the type II glucocorticoid receptor. Corticosteroids inhibit the inflammatory response to a variety of inciting agents and probably delay or slow healing. They inhibit the edema, fibrin deposition, capillary dilation, leukocyte migration, capillary proliferation, fibroblast proliferation, deposition of collagen, and scar formation associated with inflammation. [21]

There is no generally accepted explanation for the mechanism of action of ocular corticosteroids. However, corticosteroids are thought to act by the induction of phospholipase A_2 inhibitory proteins, collectively called lipocortins. It is postulated that these proteins control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes by inhibiting the release of their common precursor arachidonic acid. Arachidonic acid is released from membrane phospholipids by phospholipase A_2. [21]

The combination of levofloxacin and loteprednol eye drops was approved in Indian market on 12th July 2007 for post-operative steroid responsive inflammatory ocular conditions when ocular bacterial infections or a risk of bacterial infection exists.

1.4 INTRODUCTION TO DRUG:

1.4.1 LEVOFLOXACIN: [22-28]

- **Structural Formula:**

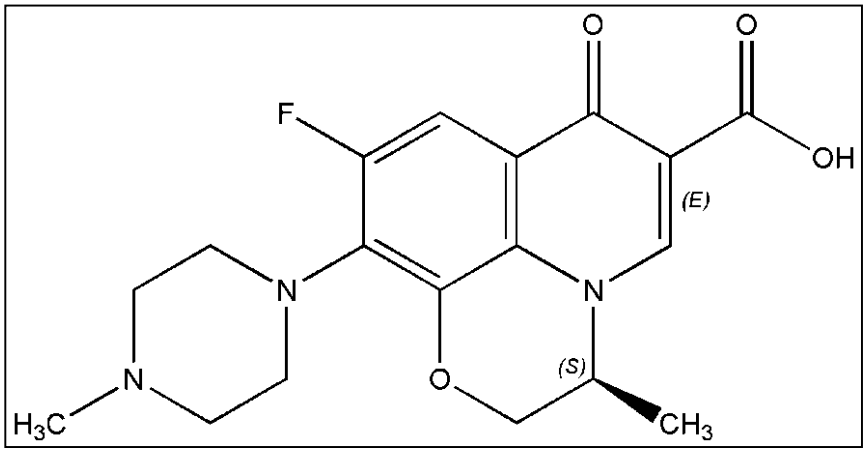

Fig. 1.2 Structure of Levofloxacin

- **Molecular Formula:** $C_{18}H_{20}FN_3O_4$
- **Molecular Weight:** 361.3675
- **IUPAC 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

- **CAS NO.:** 1 100986-85-4
- **Category:** Antibacterial and Quinolones
- **Official Status:** Official in IP-10, JP-XVI and Martine dale.

❖ **Physicochemical Properties:**

1. Appearance: A yellowish white to yellow powder.
2. Solubility: It is freely soluble in acetic acid, sparingly soluble in water and in methanol, and slightly soluble in ethanol. It dissolves in 0.1 mol/L hydrochloric acid.
3. Melting Point: About 226 ˚C (with decomposition)
4. Partition co-efficient (Log P) 0.65
5. Dissociation constant (pKa) 5.45 and 6.2
6. Storage: Store protected from light and moisture, at a temperature not exceeding 30°C.

❖ **Pharmacokinetic parameters of Levofloxacin:**

Table 1.1 Pharmacokinetic parameters of Levofloxacin

Bioavailability	99%
t_{max}	1 h
$t_{1/2}$	6-8 h
Protein binding	24-38% to plasma protein
Distribution	74-112 L/kg
Metabolism and Elimination	Mainly excreted as unchanged drug (87%); undergoes limited metabolism in humans. Mainly excreted as unchanged drug in the urine.
Drug- Drug interaction	Increased concentration of ciclosporin or tacrolimus. Reduced absorption with didanosine, ferrous sulfate or dietary supplements containing zinc, calcium, magnesium or iron. May increase plasma levels of theophylline. Increased risk of tendon rupture with corticosteroids. Reduced absorption with sucralfate and antacids containing magnesium and aluminium; administer at least 2 hr before or 2 hr after antacids. Increased half-life and decreased clearance of procainamide.
Adverse effect	Side effects include disorientation, dizziness, drowsiness, hot and cold flashes, nausea, slurring of speech, swelling and numbness in the face.

❖ **Mechanism of Action:**

Levofloxacin inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase. Levofloxacin, like other fluoroquinolones, inhibits the A subunits of DNA gyrase, two subunits encoded by the gyrA gene. This results in strand breakage on a bacterial chromosome, supercoiling, and resealing; DNA replication and transcription are inhibited.

1.4.2 LOTEPREDNOL ETABONATE [29-34]

- **Structural Formula:**

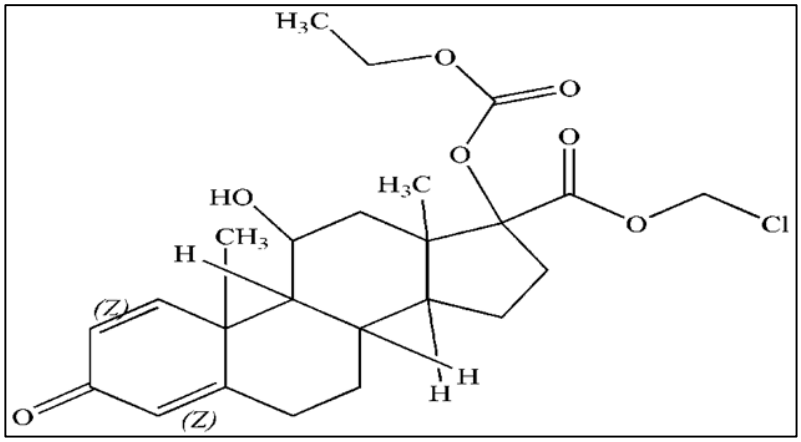

Fig. 1.3 Structure of Loteprednol etabonate

- **Molecular Formula:** $C_{21}H_{27}ClO_5$
- **Molecular Weight:** 394.889
- **IUPAC Name:** chloromethyl 17-ethoxycarbonyloxy- 11-hydroxy- 10,13-dimethyl-3-oxo-7,8,9,11,12,14,15, 16-octahydro- 6H-cyclopenta[a] phenanthrene-17-carboxylate.
- **CAS NO.:** 82034-46-6
- **Category:** Anti-inflammatory & Anti-Allergic Agents
- **Official Status:** approved in USP (after 15 may 2012)

- **Physicochemical Properties:**

1. Appearance: White to off-white powder
2. Solubility: Soluble in methanol, acetone, and methylene chloride
3. Melting Point: 220-224˚C
4. Storage: Store at 15-25°C. Don't freeze.

- **Mechanism of Action:**

Loteprednol etabonate (LE) is a "soft" steroid belonging to a unique class of glucocorticoids. Loteprednol etabonate is structurally similar to other glucocorticoids. However, the number 20 position ketone group is absent. It is highly lipid soluble which enhances its penetration into cells.

Corticosteroids are thought to act by the induction of phospholipase A_2 inhibitory proteins, collectively called lipocortins. It is postulated that these proteins control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes by inhibiting the release of their common precursor arachidonic acid. Arachidonic acid is released from membrane phospholipids by phospholipase A_2.

- **Pharmacokinetic parameters of Loteprednol etabonate:**

Table 1.2 Pharmacokinetic parameters of Loteprednol etabonate

Distribution	3.7 L/kg
$t_{1/2}$	2.8 h
Metabolism and Elimination	The drug's 17 beta-chloromethyl ester function is hydrolyzed to an inactive carboxylic acid moiety.
Bioavailability	Limited systemic exposure following topical application to the eye; plasma concentrations usually are undetectable.
Adverse effect	Include abnormal vision / blurring, burning on instillation, chemosis, discharge, dry eyes, epiphora, foreign body sensation, itching, injection, and photophobia.

1.5 INTRODUCTION TO METHOD OF UV VISIBLE SPECTROPHOTOMETERY

In multi-component formulations, the concentration of the absorbing substance is calculated from the measured absorbance using one of the following procedures [35].

(a) Assay as a single-component sample: The concentration of a component in a sample which contains other absorbing substances may be determined by a simple spectrophotometric measurement of absorbance, provided that the other components have a sufficiently small absorbance at the wavelength of measurement.

(b) Assay using absorbance corrected for interference: If the identity, concentration and absorptivity of the absorbing interferents are known, it is possible to calculate their **50**contribution to the total absorbance of a mixture [36, 37].

Based on this fact, various spectrophotometric methods are known which can be used for the analysis of combination drug samples. Following methods can be used.

- Simultaneous Equation Method (Vierodt's method)
- Derivative Spectrophotometric Method
- Absorbance Ratio Method (Q-Absorbance method)
- Dual Wavelength Method
- Ratio Spectra Derivative Method

1.5.1 Simultaneous Equation Method (Vierodt's method):

Simultaneous equation method is a quantitative method, generally used when two absorbing species in the solution have overlapping spectra. If a sample contains two absorbing drugs (X and Y) each of which absorb at the λmax of the other (as shown in figure-1.4, as λ_1 and λ_2), it may be possible to determine both drugs by the technique of simultaneous equation[38, 39].

Two equations are constructed based upon the fact that at λ_1 and λ_2, the absorbance of the mixture is the sum of the individual absorbance of X and Y.

$$Cx = (A_2 * ay_1 - A_1 * ay_2) / (ax_2 * ay_1 - ax_1 * ay_2) \text{ ..... } 1$$

$$Cy = (A_1 * ax_2 - A_2 * ax_1) / (ax_2 * ay_1 - ax_1 * ay_2) \text{ ..... } 2$$

Here, A_1 and A_2 are absorbance of the mixture at λ_1 and λ_2 nm respectively; ax_1 and ax_2 are absorptivity values of drug-X at λ_1 and λ_2 nm respectively and ay_1 and ay_2 are absorptivity values of drug-Y at the two wavelengths respectively.

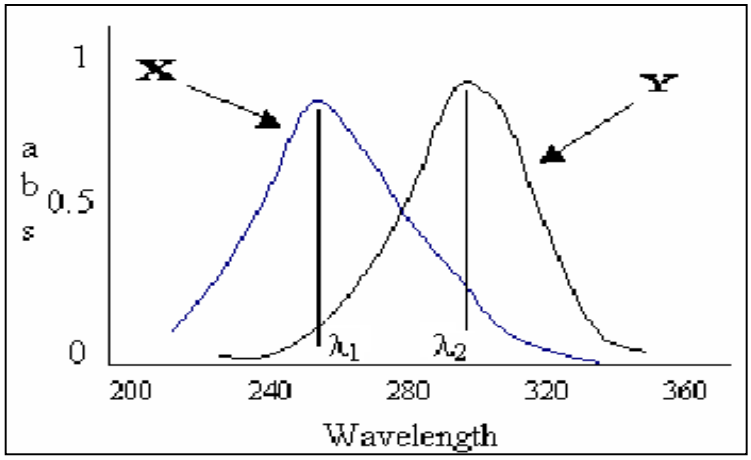

Fig. 1.4 Wavelength selection by simultaneous equation method.

1.5.2 Derivative Spectrophotometric Method:

For the purpose of spectral analysis in order to relate chemical structure to electronic transitions, and for analytical situations in which mixture contribute interfering absorption, a method of manipulating the spectral data is called derivative spectroscopy.

This involves the conversion of a normal spectrum to its first, second or higher derivative spectrum [40, 41].

1.5.3 Absorbance Ratio Method (Q-absorbance ratio method):

The absorbance ratio method is a modification of the simultaneous equation procedure. It depends on the property that, for a substance, which obeys Beer's law at all wavelengths, the ratio of absorbencies at any two wavelengths is a constant value independent of concentration or path length. In USP, this ratio is referred to as Qvalue [42].

The two wavelengths selected for this method, are the λmax of one of the component (λ_2) and a wavelength of equal absorptivities of the two components as shown in figure-1.5, i.e. an iso-absorptive point (λ_1). Two equations are constructed as described below for the Q-absorbance ratio method [43].

$$Cy = (Qm - Qx / Qy - Qx) * A_1 / ax_1 \quad \text{.....3}$$

$$Cx = (Qm - Qy / Qy - Qx) * A_1 / ay_1 \quad \text{.....4}$$

$$Qm = A_2 / A_1 \quad \text{.....5}$$

$$Q_Y = ax_2 / ax_1 \ \& \ Q_X = ay_2 / ay_1 \quad \text{.....6}$$

where, A_1 and A_2 are absorbance of sample solution at iso-absorptive point and λ_2 respectively; ax_1 and ax_2 are the absorptivities of drug-X at λ_1 and λ_2 nm respectively and ay_1 and ay_2 are the absorptivities of drug-Y at the two wavelengths respectively.

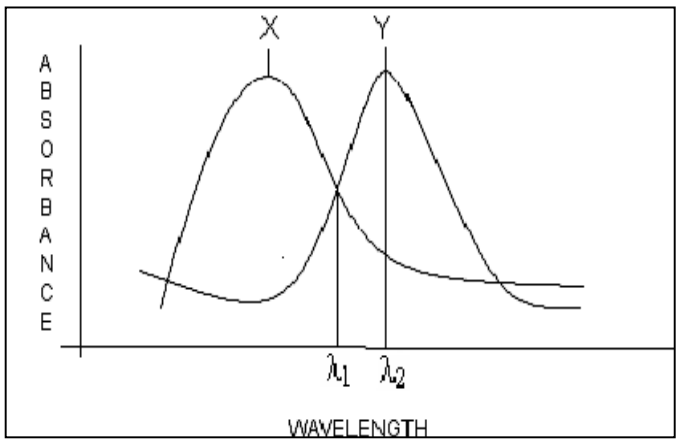

Fig. 1.5 Wavelength selection by absorption ratio method

1.5.4 Dual Wavelength Method:

The utility of dual wavelength data processing programme is to calculate the unknown concentration of component of interest present in a mixture containing both a component of interest and an unwanted interfering component. This method is based on the principle that the absorbance difference between two points on the mixture spectrum is directly

proportional to the concentration of the components of interest, independent of the interfering components.

In this method, two wavelengths are selected, where one component shows same absorbance (difference between absorbance will be zero) while the other shows significant difference in its absorbance on same wavelengths and vice versa. It involves the estimation of two components, each time considering the second one to be an interfering one [43].

1.5.5 Ratio spectra derivative method:

Salinas et al. [44] developed a new method for the analysis of binary mixtures of compounds with overlapping spectra. This method functions on two principles i.e., (i) Ratio and (ii) Derivatization.

In this method, the absorption spectra of the solutions at different concentrations of one drug are divided by the standard spectrum of another drug and the ratio spectra are obtained. Then, first derivatives of these ratio spectra are to be plotted. The amount of drug is determined by measuring the signals at λmax corresponding to a maximum or a minimum in the specified spectral region [45, 46].

1.6 INTRODUCTION TO HPLC [47-50]

HPLC was derived from classical column chromatography and has found important place in analytical techniques. This technique is based on the separation of components due to the difference in migration rate of solute through a stationary phase by a liquid mobile phase.

The parameters involved in chromatographic separation are as follows:

- Capacity factor
- Resolution
- Column efficiency
- Column selectivity
- Distribution or partition coefficient

The main components of HPLC system are high pressure pump, a column, an injector system and detector. The eluent is filtered and pumped through a chromatographic column, the sample is loaded and injected onto column, and the effluent is monitored using a detector and recorded as peaks. In HPLC assay of drugs is based on the peak area of chromatogram.

HPLC methods can be classified based on separation modes as follows:

- Liquid -Solid chromatography
- Liquid- Liquid chromatography
- Affinity chromatography
- Ion – Exchange chromatography
- Size – Exclusion chromatography
- Ion – Pair chromatography

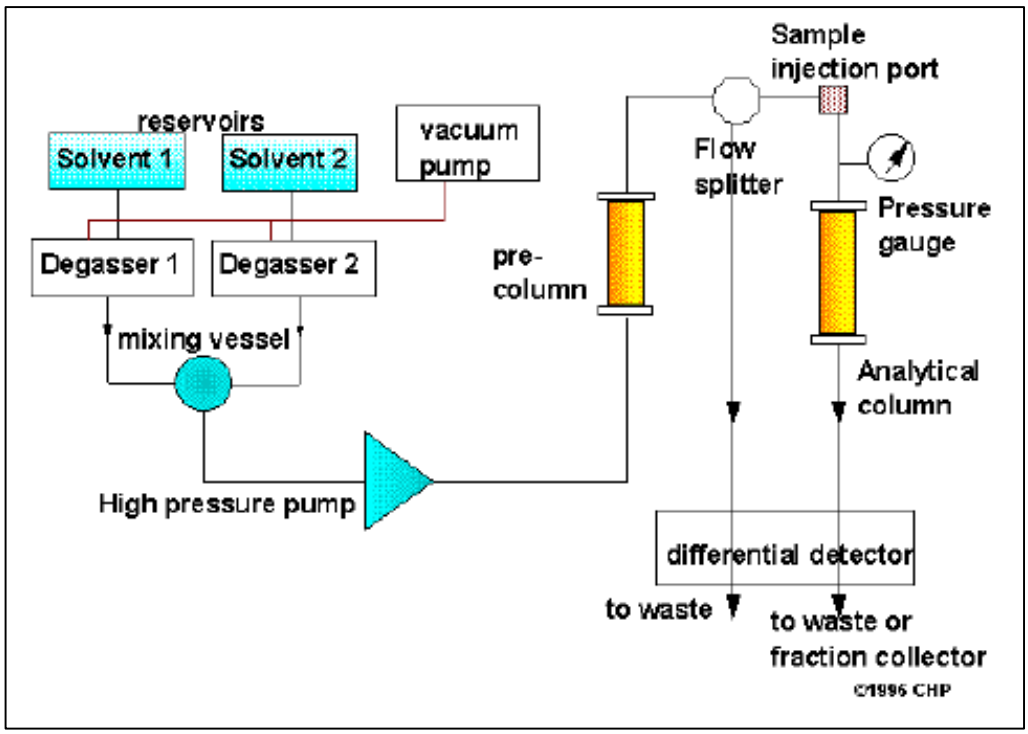

Figure 1.6 Schematic diagram of HPLC system.

1.6.1 Characteristics of HPLC method:

- Efficient, highly selective, widely applicable
- Only small sample required
- May be non-destructive of sample
- Readily adapted to quantitative analysis
- High resolving power
- Speed of separation

1.6.2 Quantitation in HPLC:

Peak height or peak area measurements only provide a response in terms of detector signal. This response must be related to the concentration or mass of the compound of interest. To accomplish this, some type of calibration must be performed.

The four primary techniques for quantitation are

- Normalized peak area method
- External standard method
- Internal standard method
- Method of standard addition

Normalized peak area method

The area percent of any individual peak is referred to the normalized peak area. This technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material and in this method; the response factor for each component is identified.

External standard method

This method includes injection of both; standard and unknown and the unknown is determined graphically from a calibration plot or numerically using response factors.

A response factor (R_f) can be determined for each standard as follows:

$$R\text{f} = \frac{\text{Standard Area (Peak height)}}{\text{Standard Concentration}}$$

The external standard approach is preferred for most samples in HPLC that do not require extensive sample preparation.

Internal standard method

The internal standard is a different compound from the analyte but one that is well resolved in the separation. The internal standard should be chosen to mimic the behavior of the sample compound. One of the main reasons for using an internal standard is for samples requiring significant pre-treatment or preparation.

Response factor is used to determine the concentration of a sample component in the original sample. The response factor is the ratio of peak areas of sample component (Ax) and the internal standard (AI_{STD}). It can be calculated using the formula,

$$R_f = \frac{Ax}{AI_{STD}}$$

On the basis of the response factor and strength of the internal standard (NI_{STD}), the amount of the analyte in the original sample can be calculated using the formula,

$$X = \frac{As}{Rf.\ AI_{STD}} \times NI_{STD}$$

Where, As is peak area of standard drug.

Method of Standard addition:

The method of standard addition can be used to provide a calibration plot for quantitative analysis. It is most often used in trace analysis. An important aspect of the method of standard addition is that the response prior to spiking additional analyte should be high enough to provide a reasonable S/N ratio (>10), otherwise the result will have poor precision.

1.7 INTRODUCTION TO METHOD DEVELOPMENT

Method of analysis is developed usually based on prior art or existing literature, using the same or quite similar instrumentation. It is rare today that an HPLC based method is developed that does not in some way relate or compare to existing, literature-based approaches. The development of any new or improved method usually tailors existing approaches and instrumentation to the current analyte, as well as to the final needs or requirements of the method. Method development usually requires selecting the method requirements and deciding on what type of instrumentation to utilize and why [51 52].

There are several valid reasons for developing new methods of analysis:

- Non-availability of a suitable method for a particular analyte in the specific sample matrix.
- Existing methods may be too erroneous, artifact, and/or contamination prone, and/or have poor accuracy or precision
- Existing methods may be too expensive, time consuming, or energy intensive, or not easily automated.
- Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest
- Newer instrumentation and techniques may have evolved that provide opportunities for improved method, including improved analyte identification or detection limits, greater accuracy or precision or better return on investment.
- There may be a need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

1.8 INTRODUCTION TO METHOD VALIDATION

1.8.1 Definition:

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product, meeting its predetermined specifications and quality characteristics [53]. Method validation is

the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

According to USFDA, validation is the process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.

1.8.2 Objective of Validation:

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Validation is documented evidence, which provide a high degree of assurance for a specific method. Any developed method may be influenced by variables like different elapsed assay times, different days, batches of reagents, instruments, equipments, environmental conditions like temperature, etc. It is expected that after the method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests reported.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH) [53], United States Food and Drug Administration (US FDA) [54], United States Pharmacopoeia (USP) [55], and World Health Organization (WHO)[56] provide a framework for performing such validations in a more efficient and productive manner.

Green et al. has given a practical guide for analytical method validation, with a description of a set of minimum requirements for a method [57]. Wegscheider et al. has published procedures for method validation with a special focus on calibration, recovery experiments, method comparison and investigation of ruggedness [58]. Seno et al. have described how analytical methods are validated in a Japanese QC laboratory [59]. The AOAC has developed a peer-verified methods validation program with detailed guidelines on exactly which parameters should be validated [60]. Winslow and Meyer recommend the definition and application of

master plan for validating analytical methods [61]. Breaux et al. have published a study on analytical methods development and validation [62].

1.8.3 Validation Parameters:

Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

The response of the analyte in test mixtures containing the analyte and all potential sample components (placebo formulation, synthesis intermediates, excipients, degradation products, process impurities, etc.) is compared with the response of a solution containing only the analyte.

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. This is sometimes termed trueness.

Accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value to the true value. National Institute of Standards and Technology (NIST) reference standards are often used; however, such a well-characterized sample is usually not available for new drug-related analytes. The extraction of the drugs from the formulation product can easily be carried out by accuracy study.

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) 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 and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

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

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

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

Detection Limit:

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.

It is the lowest analyte concentration that produces a response detectable above the noise level of the system; typically, three times the noise level. The detection limit needs to be determined only for impurity methods in which chromatographic peaks near the detection limit will be observed. The detection limit should be estimated early in the method development-validation process. It is important to test the method detection limit on different instruments, such as those used in different laboratories to which the method will be transferred.

Quantitation Limit:

The quantitation 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 levels of compounds in sample

matrices, and is used particularly for the determination of impurities and/or degradation products.

Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

This parameter is tested to establish linear relationship between analyte concentration and response of the detector. For assay methods, this study is generally performed by preparing standard solutions at five concentration levels, from 50 to 150% of the target analyte concentration. Five levels are required to allow detection of curvature in the plotted data. The standards are evaluated using the experimental conditions determined during the specificity studies.

Validating over a wider range provides confidence that the routine standard levels are well removed from nonlinear response concentrations, that the method covers a wide enough range to incorporate the limits of content uniformity testing, and that it allows quantitation of crude samples in support of process development.

Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of >0.995 is generally considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level.

Completion of linearity studies suggests the appropriate concentration range for the standards and the injection volume to be set for all subsequent studies.

Range:

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision,

accuracy and linearity. In practice, the range is determined using data from the linearity and accuracy studies. Assuming that acceptable linearity and accuracy (recovery) results are obtained as described earlier, the only remaining factor to be evaluated is precision. This precision data should be available from the triplicate analyses of spiked samples in the accuracy study.

Robustness:

Robustness of a method is its ability to remain unaffected by small but deliberate changes in parameters such as percent organic content and pH of the mobile phase, buffer concentration, temperature, and injection volume. These method parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment. Obtaining data on the effects of these parameters may allow a range of acceptable values to be included in the final method procedure. For example, if column performance changes over time, adjusting the mobile-phase strength to compensate for changes in the column may be allowed if such data are included in the validation.

Solution Stability:

Solution stability is the stability of sample test solutions in specified period of time at designated storage conditions. Routine testing involves preparation and analysis of many samples each day. It is therefore essential that solutions be stable enough to allow for delays such as instrument breakdowns or overnight analyses using auto samplers. At this point, the limits of stability should be tested. Samples and standards should be tested over at least a 48 hr period, and quantitation of components should be determined by comparison with freshly prepared standards. If the solutions are not stable over 48 hr, storage conditions or additives should be identified that can improve stability.

CHAPTER 2

LITERATURE REVIEW

TABLE 2.1: Reported analytical methods for estimation of Levofloxacin

Sr. No.	Matrix	Method	Combination with	Description	Ref.
1	Tablet	UV	Alone	Wavelength: 290 nm Solvent: 0.1M HCl Linearity: 0.25 – 12.0 µg/ml	63
2	Tablet	UV	Alone	Wavelength: 620 nm Linearity: 1.25 – 10.0 µg/ml Ion-pair complex (1:1 drug/dye) of levofloxacin with bromocresol green at pH 4	64
3	Bulk & Tablets	UV	Alone	Wavelength : 292 nm Solvent: Water: Methanol: Acetonitrile (9:0.5:0.5) Linearity : 1.0 – 12.0 µg/ml	65
4	Bulk & Tablets	UV	Alone	Wavelength : 257.4 nm Solvent : chloroform Linearity : 5 – 30.0 µg/ml	66
5	Bulk & Tablets	UV, first derivative.	Alone	Wavelength : 288 nm Solvent : Acetonitrile (10% v/v) Linearity : 2 – 12.0 µg/ml	67
6	IR Tablets	UV	Alone	Wavelength : 293.7 nm Solvent : 0.1 N Hcl Linearity : 2 – 10.0 µg/ml	68
7	Tablets	UV	Ornidazole	Wavelength : 277.5 & 319 nm Solvent : 0.1 N Hcl Linearity : 10 – 50.0 µg/ml	69
8	Tablets	UV	Ornidazole	Wavelength : 289 & 320 nm Solvent : Distilled water Linearity : 4 – 20.0 µg/ml & 8 – 40.0 µg/ml	70
9	Tablets	UV	Ornidazole	Wavelength : 293 & 277 nm Solvent : 0.1 M Hcl Linearity : 2 – 12.0 µg/ml & 2 – 32.0 µg/ml	71
10	Tablets	UV	Ambroxol	Wavelength: 219 (isoabsorptive point) & 287 nm.(λmax of Levofloxacin) Solvent : Distilled water Linearity : 2 – 14.0 µg/ml & 5 – 35.0 µg/ml	72

11	Tablets	UV	Ambroxol	Wavelength: 219 (isoabsorptive point) & 287 nm. (λmax of Levofloxacin) Solvent : Distilled water Linearity : 2 – 20.0 µg/ml & 5 – 50.0 µg/ml	73
12	Tablet	UV	Cefixime trihydrate	Wavelength : 240 & 296 nm Solvent : Methanol Linearity : 3 – 15.0 µg/ml & 3 – 15.0 µg/ml	74
13	Eye drops & Tablets	UV	Ofloxacin	Wavelength : 507 nm Solvent : distilled water Oxidation of drugs with a known excess of cerium (IV) sulphate in acidic medium Linearity : 0.5 – 3.0 µg/ml & 1.0 – 3.5 µg/ml	75
14	Tablets	UV	Gatifloxacin, Lomefloxacin	Wavelength : 426, 430, 631 nm Solvent : Double distilled water Formation of yellow and blue coloured ion-pair extractable complexes between the three drugs and two dyes, bromophenol red and fast green FCF. Linearity : 10 – 55.0 µg/ml & 10 – 70.0 µg/ml & 10 – 70.0 µg/ml	76
15	Human urine	Spectro-fluorimetry	Alone	Wavelength: Exci. 290 nm & emmi. 490 nm. Solvent: 0.1 N Hcl, water and chloroform Linearity : 50 – 600 ng/ml LOD : 1.4 ng/ml	77
16	Human urine, Tablet, Serum	Spectro-fluorimetry	Alone	Wavelength: exci. 292nm, emmi. 494 nm. Solvent: Aqueous solution containing acetic acid- sodium acetate buffer (pH 4) Linearity : 20 –3000 ng/ml LOD : 10 ng/ml LOQ : 30 ng/ml	78

17	Tablet	Spectro-fluorimetric	Garenofloxacin, Grepafloxacin	Wavelength: exci. 283 nm Solvent: Aqueous solution containing acetic acid- sodium acetate buffer (pH 4) Linearity : 0.0 – 40.0 µg/ml	79
18	Tablet	Spectro-fluorimetry	Enrofloxacin, Ofloxacin	Wavelength: exci. 359-363 nm, emmi.442-448 nm Solvent : Methanol Linearity : 50 –1000 ng/ml LOD : 17 ng/ml LOQ : 51 ng/ml	80
19	Tablet	Spectro-fluorimetry	Alone	Wavelength: Exci. 276 nm, emmi. 483 nm Solvent : Methanol Linearity : 0.04 –1.20 ng/ml LOD : 0.012 ng/ml LOQ : 0.042 ng/ml	81
20	Tablet	UV	Alone	Wavelength: 547 nm, Solvent : Distilled water Linearity : 2 –8 µg/ml LOD : 0.1475 µg/ml	82
21	Tablet	HPTLC	Alone	Stationary phase : Silica gel $60F_{254}$ prewashed with methanol Mobile phase : water-methanol-n-butanol-ammonia solution 5: 5: 5: 0.4 (v/v) Wavelength : 298 nm	83
22	Tablet	TLC with densiometry	Ambroxol hydrochloride	Stationary phase: Silica gel $60F_{254}$ Mobile phase: Chloroform: methanol: toluene: ammonia (10: 6: 3: 0.8 v/v/v/v) Wavelength : 245 nm	84
23	Tablet	HPTLC	Ornidazole	Stationary phase: Silica gel 60 F_{254} Mobile phase: n-butanol: methanol: ammonia (5:1:1.5, v/v/v)) Wavelength : 298 nm	85

24	Tablet	HPTLC	Ornidazole	Stationary phase: Silica gel 60 F_{254} Mobile phase: n-butanol: ethanol: ammonia (8 M) (5:0.5:1.5, v/v/v) Wavelength : 310 nm	86
25	Human Plasma	HPLC	Alone	Column : Intensil, C_{18} column (4.6 x 250mm x 5µm) Mobile phase: 80:20 Phosphate buffer: acetonitrile (v/v) pH 2.5 flow rate : 1 ml/min Linearity : 0.1-10 µg/ml Detector : UV Wavelength : 235 nm	87
26	Tablets	RP-HPLC	Alone	Column : 5µm Intensil, C_{18} column (4.6 x 250mm x 5µm) Mobile phase: 80:20 v/v Phosphate buffer, pH 2.5 flow rate : 1 ml/min Linearity : 0.1-10 µg/ml Detector : UV Wavelength : 235 nm	88
27	Bulk dosage form	HPLC	Alone	Column : C_{18} column (4.6 x 250mm x 5µm) Mobile phase: dihydrogen orthophosphate, methanol, acetonitrile 70:15:15 (v/v) Flow rate : 1.5 ml/min Linearity : 10-100 µg/ml Detector : UV Wavelength : 295 nm	89
28	Capsule	HPLC	Alone	Column : LUNA C_{18} column (4.6 x 250mm x 5µm) Mobile phase: 0.05 M citric acid, 1 M ammonium acetate, acetonitrile 77:1:14 v/v Flow rate : 1.0 ml/min Linearity : 10.4-104.4 µg/ml Detector : UV Wavelength : 293 nm	90

29	Cerebrospinal fluid and Plasma	HPLC	Alone	Column : C_{18} Mobile phase: 10 mmol./L KH_2PO_4: 10mmol./L $(C_4H_9)4Br$: CH_3CN (45:45:10), pH 3.0 Detector : UV Wavelength : 295 nm	91
30	Injectable Formulation	HPLC	Alone	Column: Phenomenex C_{18} column (150×4.6 mm i.d., particle size 4 µm) Mobile phase: Water : acetonitrile : phosphoric acid 0.025 M, pH adjusted to 3.0 with triethylamine (60:20:20, v/v/v) Flow rate : 1.0 ml/min Detector : UV Wavelength : 292 nm	92
31	Microspheres	HPLC	Alone	Column: ZORBAX Eclipse XDB-C_{18} column(4.6 mm×150 mm,5 µm) Mobile phase: 0.04 mol·/L phosphoric acid (adjusted to pH 4 with triethylamine) and acetonitrile (85:15). Flow rate : 1.0 ml/min Detector : UV Wavelength : 293 nm	93
32	Dosage form, Bulk & Human Serum	HPLC	Alone	Column: Nucleosil, C_{18} (10 µm, 25 cm x 0.46 cm) column Mobile phase: Water and acetonitrile (6:5) where in phosphoric acid was used to adjust the pH to 2.9 Flow rate : 1.0 ml/min	94
33	Ophthalmic gel	HPLC	Alone	Column : X TERA RP18 (4.6 x 250mm x 5µm) Mobile phase: 0.1M phosphoric acid : acetonitrile (87:13)	95

34	Human Plasma	HPLC	Alone	Column: Kromasil C_{18} column (150×4.6 mm×4 µm) Mobile phase: Acetonitrile, water, phosphoric acid and triethylamine (14: 86: 0.6: 0.3 v/v/v/v) Flow rate : 1.0 ml/min Detector : UV Wavelength : 294 nm	96
35	Plasma, bronchoalveolar lavage and bone tissues	HPLC	Alone	Column: Supelcosil (5µm, 150mm × 4.6 mm) Mobile phase: Water containing 0.4% of triethylamine, adjusted to pH 3 with concentrated orthophosphoric acid and mixed with acetonitrile (83:17, v/v). Flow rate : 1.2 ml/min Detector : UV Wavelength : 299 nm	97
36	Rat plasma	RP-HPLC	Alone	Column: Phenomenex C_{18} RP column (5 µm, 25 cm x 4.6 mm ID) Mobile phase: Acetonitrile 0.4 %, triethylamine (pH 3.1), (18:82% v/v) Flow rate : 1.0 ml/min Linearity : 20.0–5000 ng/ml Detector : UV Wavelength : 295 nm	98
37	Rat plasma and saliva	HPLC	Alone	Column : Phenomenex Luna C_{18} Column (250 × 4.6 mm×5µm) Mobile phase: Acetonitrile: water (80:20 v/v) adjusted to pH 3.5 by orthophosphoric acid Flow rate : 1.4 ml/min Linearity : 1-16 µg/ml Detector : UV Wavelength : 296 nm	99

38	Serum	RP-HPLC	Alone	Column: Phenomenex C_{18} column(5 µm, 4.6 mm ×250 mm) Mobile phase: Methanol: sodium dihydrogen phosphate buffer (adjusting to pH 2.3) (35:65) Flow rate : 1.0 ml/min Linearity : 1-16 µg/ml Detector : Fluorescence Wavelength: exci. 298 nm emmi. 491 nm	100
39	Soft tissue, bone, bile and serum	HPLC	Alone	Column: Waters Symmetry C_{18} 5 µm, 150 ×4.6 mm I.D. Mobile phase: Water, methanol and triethylamine (750: 250: 4 V: V: V) adjusted to pH 3 with orthophosphoric acid. Flow rate : 1.5 ml/min Linearity : 0.1-40 µg/ml Detector : Fluorescence Wavelength: exci. 295 nm emmi. 490 nm	101
40	Serum and urine	HPLC	Alone	Column : C_{18} Mobile phase : Acetonitrile: 0.005 M tetra butyl ammonium bromide phosphate buffer (11:1, pH 2.8) Detector : Fluorescence Wavelength: exci. 298 nm emmi. 458 nm	102
41	Human plasma and urine	HPLC	Alone	Column : Inertsil C_{18} 250 x 4.6mm; 5µm particle size Mobile phase: Copper(II) sulfate pentahydrate (5mM) containing isoleucine (10mM)-methanol (87.5:12.5, v/v) Flow rate : 1.0 ml/min Linearity : 0.08 to 5.18 µg/ml Detector : UV Wavelength: 330 nm	103

42	Tablet	RP-HPLC	Alone	Column : ACE C_{18} Column 250 x 4.6mm; 5µm particle size Mobile phase: buffer (pH 5.0) and acetonitrile in the ratio of 80:20 v/v Buffer: Dissolve 4 g of monobasic sodium phosphate dihydrate in 500 mL of water. Add 5 mL of triethylamine, and adjust with phosphoric acid to a pH of 5.9. Dilute with water to 1 L Flow rate : 1.0 ml/min Linearity : 30-90 µg/ml Detector : UV Wavelength: 294 nm	104
43	Urine	UV partial least squares calibration	Alone	Column: Shim-pack amino column Mobile phase: 25 mM potassium dihydrogen phosphate (pH adjusted to 3.1 with phosphoric acid)-acetonitrile (70:30, v/v) Flow rate : 1.0 ml/min Linearity : 0.5-16.5 µg/ml Detector : UV Wavelength: 293 nm	105
44	Bulk	HPLC	Ciprofloxacin, Moxifloxacin	Column BDS Hypersil C_{18} (100×4.6 mm, 2.4 µm) Mobile phase: Methanol/25mM phosphate buffer 28/72 (v/v) at pH 3 Flow rate : 1.0 ml/min Linearity : 0.5-16.5 µg/ml Detector : UV Wavelength: 293 nm	106
45	Tablets	RP-HPLC	Ambroxol hydrochloride	Column: Hypersil BDS C_{18} column (25cm X 4.6mm, 5µm) Mobile phase: Buffer: Acetonitrile: Methanol (650:250:100) with triethylamine and pH adjusted to 5.2 with dilute orthophosphoric acid.	107

				Flow rate : 1.0 ml/min Linearity : 7-22 µg/ml Detector : UV Wavelength: 220 nm	
46	Culture medium	RP-HPLC	Azithromycin	Column: Waters C_{18} Mobile phase: Methanol monopotasium phosphate buffer 29:71 (v/v) pH= 2.53 Flow rate : 1.0 ml/min Linearity : 10-160 µg/ml Detector : UV Wavelength: 210 nm	108
47	Micro dialysates and plasma	HPLC	Ciprofloxacin	Column: C_{18} Hypersil ODS column (150mm×2.1mm i.d., 5 µm) Mobile phase: 1.1 ml ortho-phosphoric acid was diluted with water upto 1000 ml & adjusted to pH 3.0 with 1M tetrabutylammonium hydroxide solution in water. 15 ml HPLC grade acetonitrile were filled up with this solution to 1000 ml. Flow rate : 0.4 ml/min Linearity : 10-160 µg/ml Detector : fluoroscence Wavelength: Exi. 310 nm & emmi. 467 nm	109
48	Liniment	HPLC	Dyclonine hydrochloride	Column: Zorbax SB-C_{18} (250 mm×4.6 mm, 5 µm). Mobile phase : Methanol, 0.02 mol·L^{-1} potassium dihydrogen phosphate pH 2.5 (70:30) Flow rate : 0.6 ml/min Detector : UV Wavelength: 300 nm	110
49	Gel	HPLC	Isoniazid	Column: C_{18} column Mobile phase : Acetonitrile-0.05 mol./L KH_2PO_4(18:82) Flow rate : 0.8 ml/min Detector : UV Wavelength: 262 nm	111

50	Tablets	RP-HPLC	Ornidazole	Column: C_{18} column Mobile phase: Water: acetonitrile: triethylamine (75:25:0.1, v/v) and final pH adjusted to 3.15 ± 0.02 with 5% v/v ortho-phosphoric acid Flow rate : 1.5 ml/min Detector : UV Wavelength: 310 nm	112
51	Tablets	RP-HPLC	Ornidazole	Column: Phenomenex Luna C_{18} column (5µ, 150 x 4.6mm I.D). Mobile phase : Triethylamine (0.5% v/v adjusted to pH 3 using orthophosphoric acid), acetonitrile and methanol (40:30:30) Flow rate : 0.5 ml/min Detector : UV Wavelength: 310 nm	113
52	Tablets	HPLC	Ornidazole	Column: Phenomenex C_{18} column (250 mm 4.6 mm id, 5 µm) Mobile phase: KH_2PO_4 buffer (pH 6.8): methanol: acetonitrile (70: 15: 15, v/v/v) Flow rate : 1.5 ml/min Detector : UV Wavelength: 295 nm	114
53	Tablets	HPLC	Ornidazole	Column: Hypersil BDS C_{18} 150mm × 4.6mm × 5µm column Mobile phase: Buffer: Acetonitrile (75:25) Buffer: Transfer 1.0 ml of triethylamine into 500 ml of distilled water and adjust pH with ortho phosphoric acid to 3.15. Flow rate : 1.0 ml/min Detector : UV Wavelength: 315 nm	115

54	Human plasma	HPLC	Zidovudine	Column: Octadecylsilane column (150 × 4.6 mm × 5 µm) Mobile phase: 86 : 14 v/v 25 mM sodium phosphate monobasic monohydrate and 0.1% trifluoroacetic acid (pH 2.4) , acetonitrile Detector : UV Wavelength: 266 nm	116
55	Human urine	HPLC	Ceftriaxone sodium, Metronidazole	Column: Kromasil C_{18} (250 mm × 4.6 mm, 5 µm Mobile phase: 1.5 mM KH_2PO_4 (pH 4.5) with 0.0125% triethylamine, methnol (70:30, v/v) Detector : UV Wavelength: 300 nm	117
56	Human urine	HPLC	Garenoxacin, Moxifloxacin	Column: C_{18} column Mobile phase: acetonitrile/0.1 M phosphoric acid/ sodium hydroxide buffer (pH 3.0)/0.01 M n-octylamine (pH 3.0) Flow rate : 1.0 ml/min Detector : UV Wavelength: 292 nm	118
57	Tablets & injection preparation	HPLC	Gatifloxacin, Lomefloxacin and Pefloxacin	Column: LiChrospher RP-18 column (5µm, 125mm×4 mm) Mobile phase: Water: acetonitrile (80:20, v/v) with 0.3% of triethylamine and pH adjusted to 3.3 with phosphoric acid Flow rate : 1.0 ml/min Detector : UV Wavelength: 295 nm	119
58	Human plasma	HPLC	Gatifloxacin, Sparfloxacin and Moxifloxacin	Column: Kromasil C_{18} column Mobile phase: phosphate buffer (pH 2.5) acetonitrile (80:20, v/v) Linearity : 100-10000 ng/ml LOQ : 100 ng/ml	120

59	Serum	HPLC	Gatifloxacin and Moxifloxacin	Column: Supelcosil Plus (150mm × 4.6mm× 5µm) Mobile phase: 10mM phosphate buffer (pH 2.5), acetonitrile (88:12, v/v) and 2mM tetrabutyl ammonium bromide Flow rate : 1.0 ml/min Detector : fluoroscence Wavelength: exci. 296 nm emmi. 504 nm	121
60	Tablet	RP-HPLC	Lomefloxacin Hydrochloride, Gatifloxacin and Sparfloxacin	Column: Chromolith Performance RP-18e (100 x 4.6 mm× 5µm) Mobile phase: Methanol: 0.025M KH_2PO_4 adjusted to pH 3 using ortho - phosphoric acid (20:80,v/v) Flow rate : 4.0 ml/min Detector : UV Wavelength: 290 nm	122
61	Human Serum	HPLC	Pazufloxacin, Ciprofloxacin	Column: C_8 column Mobile phase: 1% triethylamine (pH 3.0) /acetonitrile (86/14, v/v)	123
62	Human Plasma	HPLC	Ciprofloxacin, Gatifloxacin, Moxifloxacin, Trovafloxacin and Cinoxacin	Column: C_8 column Mobile phase: 25 mM phosphate with 50% acetonitrile & adjusted pH to 3.0 Flow rate : 1.0 ml/min Detector : fluoroscence Wavelength: exci. 293 nm emmi. 500 nm	124
63	Mouse tissues and plasma	LC-MS	Isoniazid, Rifampicin	Column: C_4 column (250mm × 4.6mm ×5.0mm) Mobile phase: 0.05% formic acid and methanol (93:7,v/v) Flow rate : 1.0 ml/min Detector : Mass spectrometry	125

64	Catheter segments from a mouse model	LC-ESI-MS	Rifampicin	Column: Waters C_{18} column (2.1mm×100 mm, 3.5µm) Mobile phase: Mobile phase A consisted of acetonitrile, water, formic acid (3:97:0.2, v/v/v) and mobile phase B consisted of acetonitrile, formic acid (100:0.2, v/v). Flow rate : 0.4 ml/min Detector : mass spectrometry	126
65	Urine	Micellar liquid chromatography method with fluorescence detection	Alone	Column: C_8 column Mobile phase: 0.15M sodium dodecyl sulphate, 12.5% propanol and 0.5% triethylamine at pH 3.0 Flow rate : 1.0 ml/min Detector : fluoroscence Wavelength: exci. 285 nm emmi. 465 nm	127
66	Tablet	HPLC	Alone	Column: ACE C_{18} 250mm × 4.6mm ID × 5µm particle size Mobile phase: Mixture of 0.5% (v/v) triethyl amine in sodium dihydrogen orthophosphate dihydrate (25mM; pH 6.0) and methanol Detector : UV Wavelength: 294 nm	128
67	Plasma and Amniotic Fluid	HPLC	Levofloxacin Moxifloxacin & Other Quinolones	Mobile phase : 15 mM citrate buffer, pH 3.2, 9% Acetonitrile, 5% Methanol, 5 mM TMAB Flow rate :1.5 ml/min	129
68	Tablet	HPLC	four novel fluoroquinolone	Column: Zorbax Eclipse XDB C_{18} Mobile phase: Mobile phase A [0.1%, v/v, aqueous Trifluoro acetic acid] and mobile phase B [Methanol: Acetonitrile: Trifluoro acetic acid (85:15:0.1, v/v/v)] Detector : UV Wavelength: 285 nm	130

69	Intravenous infusion	HPLC	5-HMF (5-Hydroxymethyl furfural)	Column: LichroSphere C_{18} column 150mm x 4.6mm, 5µm Mobile phase: pH 3.0 buffer (0.04M, ortho phosphoric acid buffer, adjusted pH 3.0 with triethylamine) - acetonitrile (87:13, v/v) Detector : UV Wavelength: 284 nm	131
70	Human Serum	HPLC with fluorescence detection	Pazufloxacin, Ciprofloxacin	Column: C_8 column Mobile phase: 1% triethylamine (pH 3.0)/acetonitrile (86/14, v/v). Flow rate : 1.0 ml/min Detector : fluoroscense Wavelength: exci. 300 nm emmi. 450 nm	132
71	Rat blood and bile	HPLC	Alone	Column: C18 column 150mm x 4.6mm, 5µm Merck LiChrospher Mobile phase: Acetonitrile, 1 mM 1-octanesulfonic acid (40:60, v/ v, pH 3.0 adjusted with orthophosphoric acid Flow rate : 1.0 ml/min Detector : fluoroscence Wavelength: exci. 292 nm emmi. 494 nm	133
72	Human Serum	HPLC	Alone	Column: Inertsil ODS-2 column Detector : fluoroscense Wavelength: exci. 290 nm emmi. 500 nm	134
73	Tablets	HPLC	Moxifloxacin	Column: Nucleosil 100-5C18 Nautilus column (125 × 4 mm i.d., 5 µm) Mobile phase: acetonitrile and 0.01 mol/L sodium dihydrogen phosphate at pH 2.7 (3:97, v/v) and of acetonitrile, 0.01 mol/L sodium dihydrogenphosphate at pH 2.7 (50:50, v/v) Flow rate : 1.5 ml/min	135

				Detector : fluoroscense Wavelength: exci. 295 nm emmi 440 nm	
74	Human Urine	Adsorptive square-wave anodic stripping voltammetry	Alone	Using square-wave anodic stripping voltammetry and accumulation at +0.4 V versus Ag/AgCl (saturated KCl) for 300s, Linearity 6.0×10^{-9} to 5.0×10^{-7} M levofloxacin. LOD : 5.0×10−9 M.	136
75	Bulk & tablets	Bromatometric Estimation	Lomefloxacin HCl and Sparfloxacin	Solvent : Bidistilled water bromate-bromide as the oxidimetric reagent determined by treating with fixed amount of either methylene blue, methyl orange or thymol blue then measuring absorbances at 678 nm, 510 nm 545 nm Linearity : 0.1-1.0 µg/ml	137
76	Human Urine	Capillary Electrophoresis	Alone	Mobile phase: 50 mM phosphate buffer at pH 2.30 Detection limit of 10 nM when the sample was prepared in deionized water linear ranges of levofloxacin in deionized water and untreated urine were 10^{-7} to 5×10^{-3} M	138
77	Tablets	Capillary zone electrophoresis	Ciprofloxacin, Gatifloxacin, Moxifloxacin	Buffer: Tris/hydrochloride and sodium tetraborate buffer Mixture Separation time: 3 minutes	139
78	Human urine	Flow injection chemiluminescence	Alone	Linearity: 0.01–1.44 µg/mL for levofloxacin LOD : 8 ng/ml	140
79	Tablet	Extractive colorimetric	Alone	Formation of colored chloroform extractable ion-pair complexes (1:1 and 1:2 drug/dye) of levofloxacin with bromophenol blue (BPB) and bromocresol green (BCG) in aqueous acidic medium Absorbance maxima at 424 and	141

				428 nm for LVFX-BPB and LVFX-BCG	
80	Aqueous Humour and Tablets	RP-UPLC	Alone	Column: Waters Acquity HSS T-3 (100 × 2.1 mm, 1.8 μm) Run time : 5 minute Mobile phase: mixture of A: 0.1% aqueous TFA and B: acetonitrile, delivered at a constant Flow rate of 0.45 mL/min. Detector :UV Wavelength : 288 nm	142
81	Pharmaceutical preparations	Flow injection analysis using UV detection, potentiometry and conductometry	Alone	Solvent: 0.2 M acetate buffer at pH 3 having 10% MeOH. Flow rate of 1 ml min^{-1} Wavelength: 288 nm LOD for FIA : 3×10^{-7} M (S/N=3) LOQ for FIA: 1×10^{-7} M (S/N=10)	143
82	Tablets	Pulse stripping voltammetry and chemometrics	Alone	Deposition time: 80 seconds Deposition potential of 1250 mV Scan rate: 25 mV/s	144
83	Dosage form	Resonance light scattering determination	Alone	Buffer: CH_3COOH CH_3COONa buffer solution of pH 4.5. Wavelength: 468 nm Linearity: 0.059–22.4 l gmL^{-1}.	145
84	Dosage form	Rapid chiral separation and impurity determination	Alone	Column: C_{18} column mobile phase: Methanol water solution (containing10 mmol L^{-1} l-leucine and 5 mmol L^{-1} copper sulfate) (88:12, v/v) flow-rate: 1.0 $mLmin^{-1}$ linearity : 0.5-400 mg/L	146
85	Catheter segments from a mouse model	LC-ESI-MS	Rifampicin	Column : Symmetry C_{18} column (2.1mm×100 mm, 3.5m; Waters, Milford, MA) Mobile phase: Mobile phase A consisted of acetonitrile: water:	147

				formic acid (3:97:0.2, v/v/v) and mobile phase B consisted of acetonitrile: formic acid (100:0.2, v/v). linearity concentration range of 0.02–2 µg/g	
86	Human plasma	Hydrophilic interaction liquid chromatography-tandem mass spectrometry	Alone	Column : Atlantis HILIC Silica Mobile phase : acetonitrile-ammonium formate (100 mM, pH 6.5) (82:18 v/v) electrospray ionization mode Linearity: 10.0–5000 ng/ml.	148

TABLE 2.2: Reported analytical methods for estimation of Loteprednol

Sr. No.	Matrix	Method	Combination with	Description	Ref
1	Eye drops	HPLC	Alone	Column: Phenomenex Prodigy Phenyl 3 (5µm, 4.6 x 150mm) Mobile phase: 9:1 acetic acid (0.25%): methanol (mobile phase A) and acetonitrile (mobile phase B). Detector : UV Wavelength : 244 nm	149
2	API	HPLC	Alone	Column: Phenylsilica (Alltima Phenyl, 250 mm, 4.6 mm i.d. Mobile phase: Mixture of water, acetonitrile and acetic acid (57.0: 42.5: 0.5 v/v %) Flow rate :1.8 ml/min	150
3	API	HPLC	Alone	Column : Alltech Alltima phenyl column 4.6 mm×250 mm×5µm Mobile phase: Acetonitrile/Water/Acetic acid as solvent system with a ratio of 42.5: 57: 0.5. Flow rate: 1.9 ml/min	151

4	Eye drops	HPLC	Alone	Column: Nova-Pak phenyl (75 × 3.9 × 4 µm) Mobile phase: Acetonitrile: water: acetic acid 45:54:1 Flow rate: 1 ml/min Injection volume: 20 µL Detector: UV 254 nm	152

CHAPTER 3

AIM & OBJECTIVES

3.1 AIM OF PRESENT WORK

Pharmaceutical analytical procedures may be used for identification and quantitative analysis of the active moiety in the sample of drug substances or products. In the past few decades, number of new chemical entities and newer formulation of the known entities have been introduced into the market. Multicomponent dosage forms are also introduced as they are known to be beneficial. The development of an assay procedure for such dosage forms poses considerable challenges to the analytical chemist owing to complexity of these dosage forms as it contain multiple drug entities.

Several methods have been reported for the individual estimation of Levofloxacin and Loteprednol and their combination with other drugs.

Till date, not a single method has been developed for combination of Levofloxacin and Loteprednol and hence it was endeavored to develop UV Spectrophotometric and RP-HPLC method for simultaneous estimation of drugs in combined dosage form.

3.2 OBJECTIVES OF PRESENT WORK

- To develop UV-Visible spectrophotometric & RP-HPLC methods for simultaneous estimation of Levofloxacin and Loteprednol in their combined dosage form.
- To validate the developed methods in terms of Linearity, Range, Accuracy, Precision, Limit of Detection, Limit of Quantification, Robustness, and Specificity as per ICH guidelines.
- To apply the developed method for the quantification of Levofloxacin and Loteprednol from their marketed dosage forms.
- Comparison of the developed methods.

CHAPTER 4

IDENTIFICATION OF DRUG

4. IDENTIFICATION OF DRUGS:

Identification of drugs was carried out by melting point, UV-Visible Spectroscopy and IR spectroscopy.

❖ **Instrumentation:**

- Melting Point Apparatus- T603160, (EIE Instruments, Pvt. Ltd.)
- UV/Vis-2400, Version-2.21 double beam spectrophotometer (Shimadzu, Columbia, MD)
- JASCO FT/IR-6100, (Inc. Japan) TGS Detector with Spectra Manager Software.

4.1. Melting Point Determination:

Melting point of Levofloxacin and Loteprednol etabonate has been determined using melting point apparatus. The melting point of the pure drugs was taken by open capillary method.

Table 4.1 Melting Point of Levofloxacin & Loteprednol etabonate

Drug	Reported Melting Point (^{o}C) [153-154]	Observed Melting Point (^{o}C)
Levofloxacin	225-227	226-228
Loteprednol etabonate	220-224	220-223

4.2. FT-IR Spectra Determination:

IR Spectra of pure drugs was taken using FT-IR spectrophotometer.IR spectra obtained was verified with the reported IR spectra available in literature.

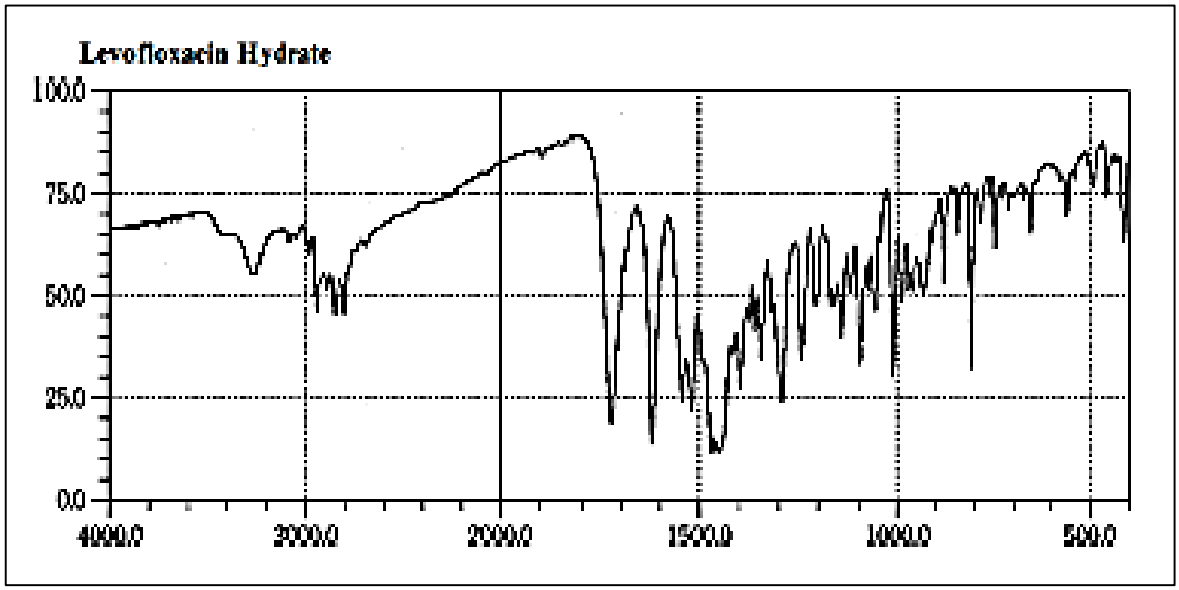

Fig. 4.1 Reported FT-IR Spectra of Levofloxacin [155]

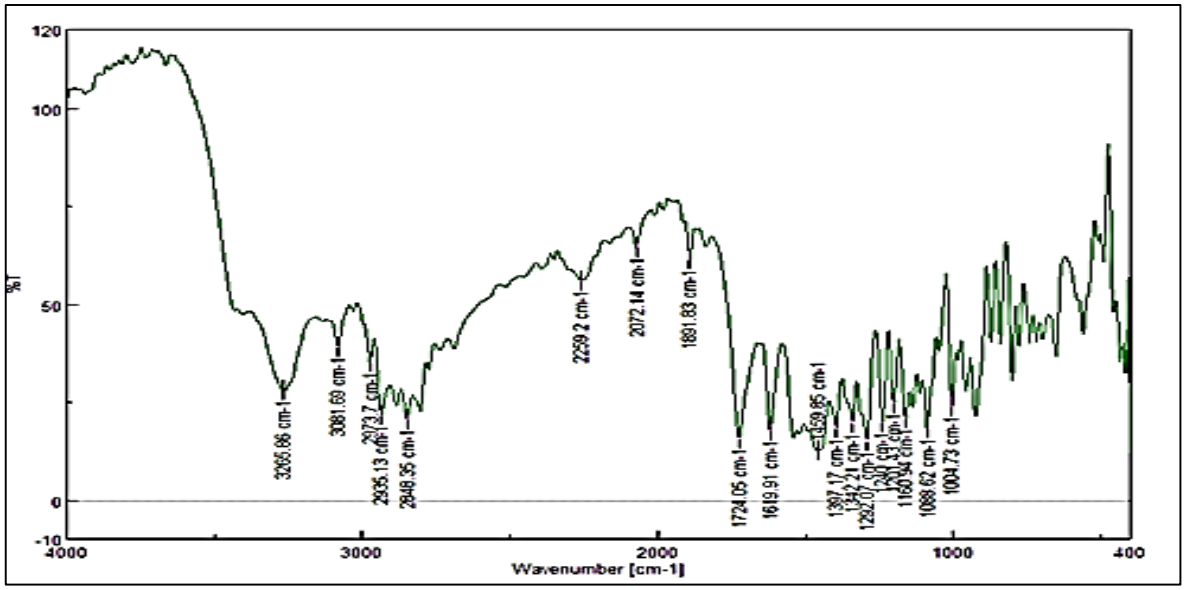

Fig. 4.2 Recorded FT- IR Spectra of Levofloxacin

Table 4.2 Observed FT-IR Peaks of Levofloxacin

Functional Group	Observed FT-IR Peaks (cm^{-1})	Functional Group	Observed FT-IR Peaks (cm^{-1})
O-H stretching	3263	Aromatic C=C stretching	1619
Aromatic C-H stretching	3082	C-O stretching	1397
C-H stretching in $–CH_3$	2973	C-N stretching	1360
C-H stretching in $–CH_3$	2937	C-O stretching	1291
C-H stretching in $–CH_3$	2848	C-F stretching	1208
C-H stretching in $–CH_3$	2803	C-O-C stretching	1135
C=O stretching	1725	C-O stretching	1241
C-H out of plan bending	742	Aromatic C=C bending	670

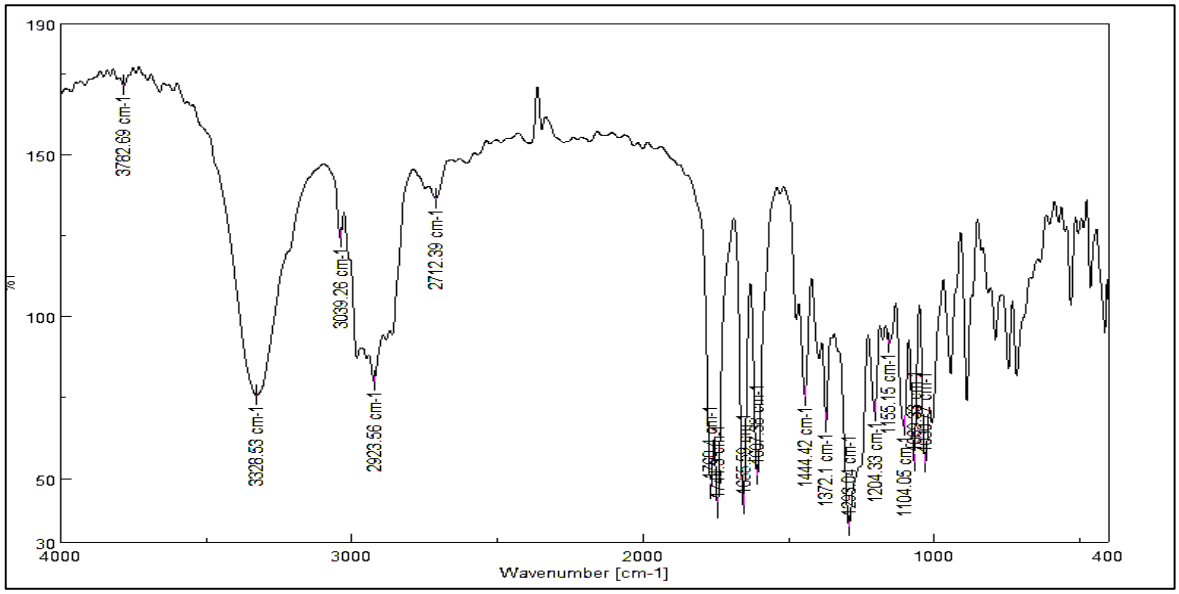

Fig. 4.3 Recorded FT IR Spectra of Loteprednol etabonate

Table 4.3 Reported and Observed FT-IR Peaks of Loteprednol etabonate

Functional Group	Observed FT-IR Peaks (cm^{-1})	Functional Group	Observed FT-IR Peaks (cm^{-1})
C-H stretching Aromatic	3039	C=C stretching Alkene	1655
C-H stretching Aliphatic	2924	C-H Bending	1444
COO stretching	1768	O-H Bending	1372

CHAPTER 5

EXPERIMENTAL WORK

5.1 EXPERIMENTAL WORK:

5.1.1 Instrumentation:

- UV-Visible Double-Beam spectrophotometer; Model UV-2450 PC series, Shimadzu Inc. Japan, Wavelength range: 200.00 to 800.00 nm.
- Analytical Balance; Model: CX -220, Citizen.
- Ultra Sonicator; Model TRANS-O-SONIC; D-compect.

5.1.2 Materials and Methods:

- API Levofloxacin - Gift sample from Torrent Research Centre
- API Loteprednol etabonate – Sample was purchased from Ajanta Pvt. Ltd.
- Methanol (AR Grade, S.D. Fine Chemicals Ltd., Bombay, India)

5.1.3 Preparation of Standard Stock Solution & Sample Solution:

Standard Stock Solution of Levofloxacin (100 µg/mL) & Loteprednol etabonate (100 µg/mL):

Levofloxacin (25 mg) and Loteprednol etabonate (25 mg) was weighed accurately and transferred to individual 25 mL amber colored volumetric flasks and dissolved in methanol. The solution was sonicated for 5 minutes. The flasks were shaken and volume was made up to the mark with methanol to give solution containing 1000 µg/mL of Levofloxacin and Loteprednol etabonate. Aliquot of 1 mL was pipetted out from stock solution of Levofloxacin & Loteprednol etabonate (1000 µg/mL) and transferred to 10 mL amber colored volumetric flask. The volume was made with methanol to obtain final concentration of 100 µg/mL of Levofloxacin & Loteprednol etabonate.

Preparation of sample solution from eye drops:

Formulation is not available in India. Eye drops contain 0.5% w/v of Loteprednol Etabonate and 1.5% w/v of Levofloxacin. Synthetic mixture was prepared. Sample solution was prepared by diluting 0.1 mL of eye drops to 10 mL with methanol in amber coloured volumetric flask. From that, 1 mL of aliquot was diluted to 10 mL with methanol, which

correspondingly gives 5 µg/mL and 15 µg/mL concentration of Loteprednol Etabonate and Levofloxacin respectively.

Levofloxacin was quantified using following equation:

$$A = a\ b\ c$$

Where, a= absorptivity

b= path length

c= concentration gm/100 mL

Loteprednol etabonate was quantified using following equation:

$$Cx = Qm - Qy / Qx - Qy \ . \ A_1 / a_{x1}$$
$$C_Y = Qm - Qy / Qy - Qx \ . \ A_1 / a_{x1}$$

In the quantitative assay of two components in admixture by the absorbance ratio method, absorbance are measured at two wavelengths, one being the λ max of one of the components (λ_2) and the other being a wavelength of equal absorptivity of the two components (λ_1), an iso-absorptive point.

5.1.4 Preparation of Solution for Method Validation:

5.1.4.1 Preparation of Linearity Curve for both Levofloxacin & Loteprednol:

Appropriate volume of aliquot 0.5, 1.0, 1.5, 2.0 and 2.5 mL was pipetted out from 100 µg/mL standard solution of Levofloxacin & Loteprednol were transferred to individual 10 mL amber colored volumetric flask. The flasks were shaken and volume was made upto the mark with methanol to obtain final concentration of 5, 10, 15, 20 & 25 µg/mL of both the drugs.

5.1.4.2 Precision:

The precision of analytical method is the degree of agreement among individual results when the method is applied to multiple sampling of homogenous samples. It provides an indication of random error in results and was expressed as coefficient of variance (CV).

A) Intra-day and Interday precision:

Intra-day and Inter-day precision was determined by measuring the absorbance of Levofloxacin & Loteprednol three times within a day and on three different days, respectively. For Intraday and Interday precision 0.5, 1.5 & 2.5 ml were taken from working standard solution of 100 µg/mL in 10 mL volumetric flasks & volume was made upto 10mL with methanol to obtain concentration 5, 15 and 25 µg/ml respectively for both the drugs.

Absorbance of each solution was measured three times in a day for intraday precision and at three different days for interday precision at 269.29 and 298.5 nm for Levofloxacin.

Absorbance of each solution was measured three times in a day for intraday precision and at three different days for interday precision at 269.29 for Loteprednol etabonate.

B) Repeatability:

It is a measure of precision under the same operating conditions over a short interval of time. It is sometimes referred to as intra-assay precision. To study the repeatability, six determinations at 15 µg/mL concentration of Levofloxacin and Loteprednol etabonate were carried out. Absorbances of solutions of Levofloxacin were measured at 269.29 & 298.5 nm. Solutions of Loteprednol etabonate were carried out at 269.29 nm.

5.1.4.3 Limit of Detection

The calibration curve was repeated six times and the standard deviation of intercepts was calculated for both Levofloxacin & Loteprednol. LOD was calculated using following equation.

$$\mathbf{LOD = 3.3\ \sigma/s}$$

Where, σ = standard deviation of intercepts

s = slope of straight line.

5.1.4.4 Limit of Quantification:

The calibration curve was repeated six times and the standard deviation of intercepts was calculated for both Levofloxacin & Loteprednol. LOD was calculated using following equation.

$$\mathbf{LOD = 10\ \sigma/s}$$

Where, σ = standard deviation of intercepts

s = slope of straight line.

5.1.4.5 Robustness:

Robustness was performed on concentration (15µg/mL) of Levofloxacin and Loteprednol etabonate. Robustness of the method was determined by making change in λ_{max} of the drug by ±2 nm. The % Assay values were calculated and compared with that of standard. Results were reported in terms of % RSD.

5.2 RESULT & DISCUSSION:

5.2.1 Selection of Solvent & Wavelength:

Due to solubility of Levofloxacin and Loteprednol etabonate in Methanol, it was selected as a solvent.

From the obtained spectrum of Levofloxacin and Loteprednol etabonate, 298.5 nm was selected as one of the wavelength for the estimation of Levofloxacin because at 298.5 nm Loteprednol shows almost zero absorbance.

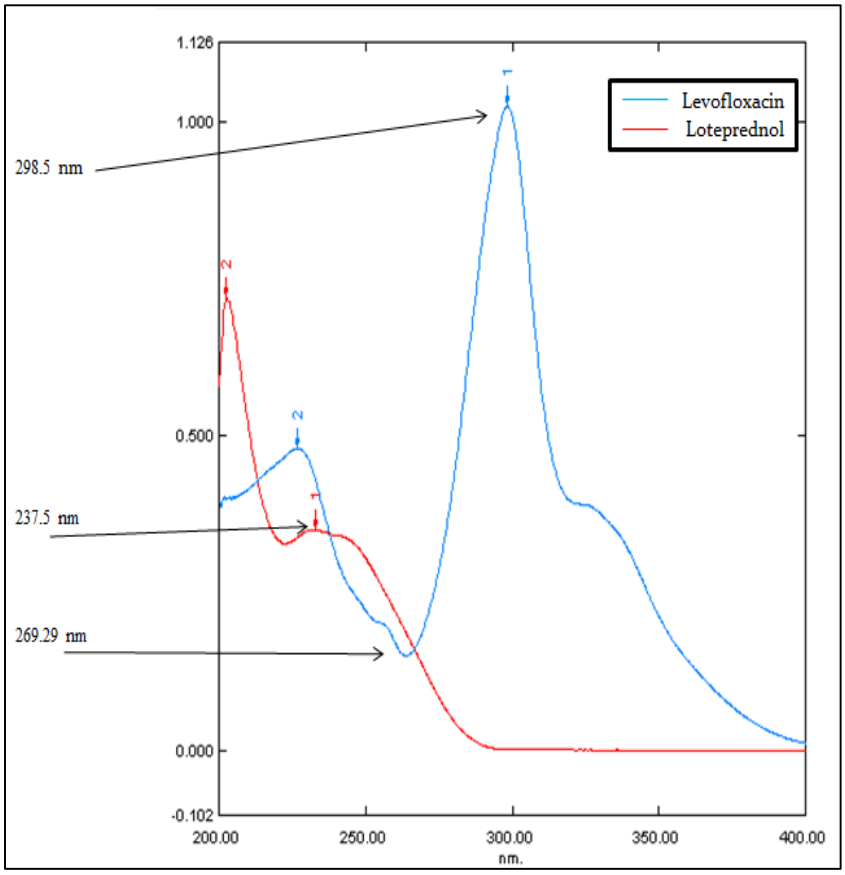

Fig 5.1 Overlay Spectra of 10 µg/mL Levofloxacin & Loteprednol etabonate in methanol

5.2.2.1 Validation Parameters:

Linearity:

Linearity range was found to be 5-25 µg/mL for Levofloxacin in methanol with correlation co-efficient 0.998.

Table 5.1 Linearity of Levofloxacin (5-25 µg/ml) at 298.5 nm

Conc. (µg/ml)	Mean Response ± S.D. *	% R.S.D.
5	0.531 ± 0.0039	0.8394
10	1.126 ± 0.0116	1.0349
15	1.591 ± 0.0101	0.6392
20	2.193 ± 0.0068	0.3106
25	2.741 ± 0.0096	0.3517
Linearity Equation		y = 0.1097x - 0.0092
Correlation Coefficient		R^2 = 0.998
Slope		0.1097
Intercept		-0.009

* n= 6

Absorptivity: 1065.188

Table 5.2 Linearity of Levofloxacin (5-25 µg/mL) at 269.29 nm

Conc. (µg/ml)	Mean Response ± S.D. *	% R.S.D.	Absorptivity
5	0.102 ± 0.0013	1.2967	205.00
10	0.200 ± 0.0034	1.7029	200.00
15	0.290 ± 0.0039	1.3561	193.33
20	0.389 ± 0.0060	1.5545	194.50
25	0.501 ± 0.0083	1.6636	200.40
Linearity Equation		y = 0.019x + 0.000	
Correlation Coefficient		0.998	
Slope		0.019	
Intercept		0.000	

n=6

Absorptivity: 198.64

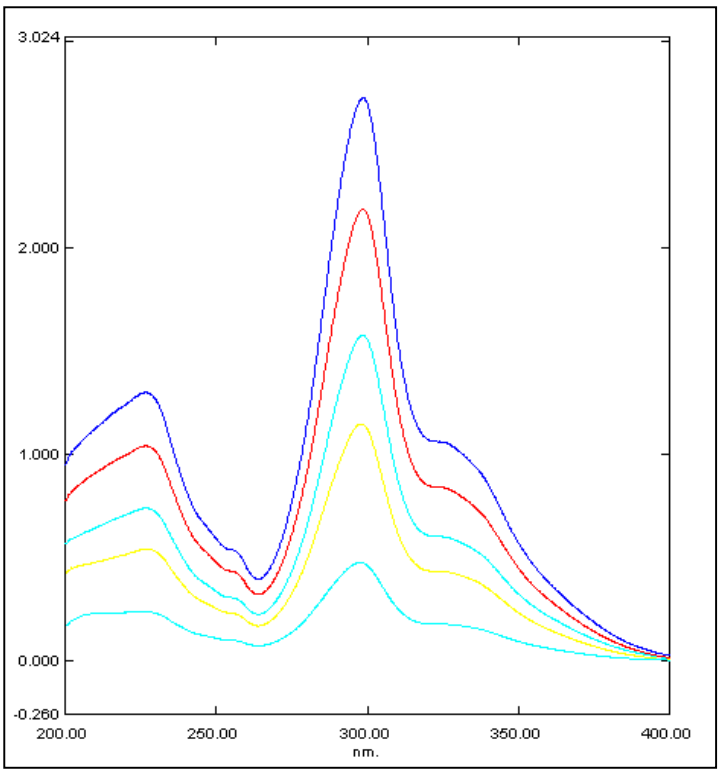

Fig. 5.2 Linearity Overlay Spectra for Levofloxacin

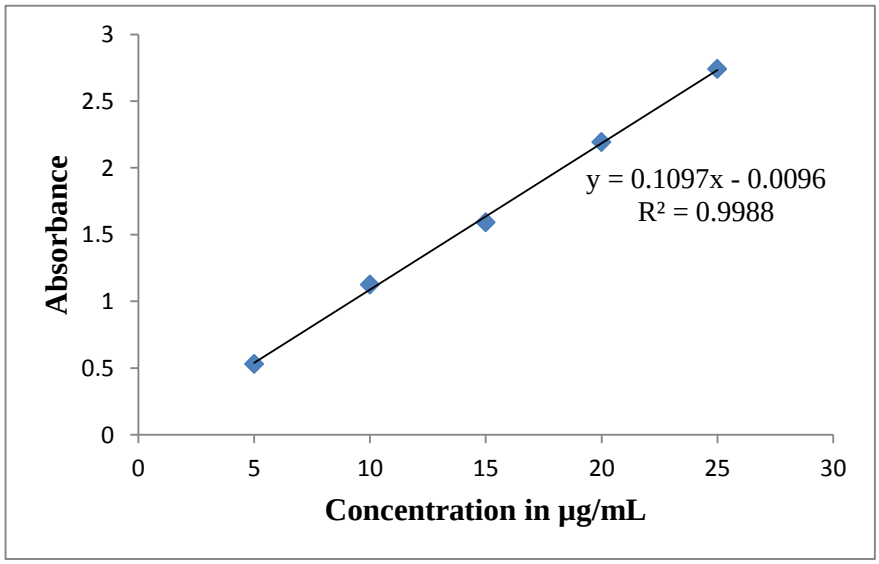

Fig. 5.3 Linearity Curve for Levofloxacin at 298.5 nm

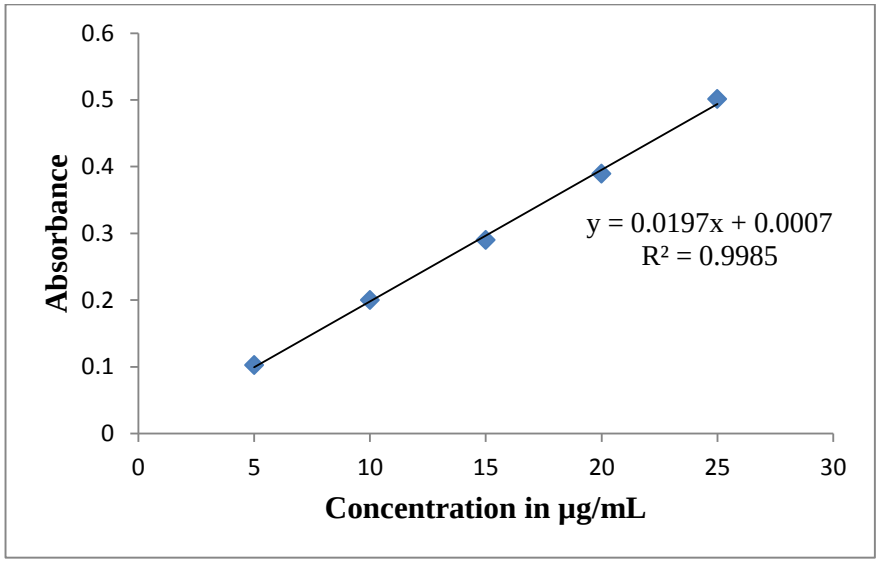

Fig. 5.4 Linearity Curve for Levofloxacin at 269.29 nm

5.2.2.2 PRECISION

A) Interday Precision:

Table 5.3 Interday Precision of Levofloxacin

Conc. (µg/ml)	Abs.	Conc. (µg/ml)	Mean Abs. ± S.D.	Mean Conc. (µg/ml) ± S.D.	% R.S.D.	Abs.	Conc. (µg/ml)	Mean Abs. ± S.D.	Mean Conc. (µg/ml) ± S.D.	% R.S.D.
	269.29 nm					298.5 nm				
5	0.101	5.08	0.101 ± 0.0015	5.09 ± 0.0764	1.4986	0.529	4.96	0.532 ± 0.0031	4.99 ± 0.0280	0.5602
	0.100	5.03				0.533	5.00			
	0.103	5.18				0.535	5.02			
15	0.304	15.18	0.308 ± 0.0050	15.30 ± 0.2500	1.6339	1.601	15.03	1.593 ± 0.0064	14.96 ± 0.0605	0.4043
	0.309	15.55				1.589	14.91			
	0.299	15.05				1.591	14.93			
25	0.495	24.92	0.497 ± 0.0044	25.02 ± 0.2179	0.9711	2.667	25.03	2.662 ± 0.0044	24.99 ± 0.0413	0.1653
	0.502	24.57				2.660	24.03			
	0.494	24.87				2.659	24.96			

B) Intraday Precision:

Table 5.4 Intraday Precision of Levofloxacin

Conc. (µg/ml)	Abs.	Conc. (µg/ml)	Mean Abs. ± S.D.*	Mean Conc. (µg/ml) ± S.D.*	% R.S.D.	Abs.	Conc. (µg/ml)	Mean Abs. ± S.D.*	Mean Conc. (µg/ml) ± S.D.*	% R.S.D.
	269.29 nm					298.5 nm				
5	0.099	4.98	0.101 ± 0.0020	5.08 ± 0.1000	1.9685	0.532	4.99	0.531 ± 0.0252	4.99 ± 0.0252	0.5058
	0.103	5.18				0.529	4.96			
	0.101	5.08				0.534	5.01			
15	0.298	15.00	0.301 ± 0.0350	15.18 ± 0.1756	1.1565	1.598	14.99	1.598 ± 0.0115	15.00 ± 0.1083	0.7222
	0.302	15.20				1.610	15.11			
	0.305	15.35				1.587	14.88			
25	0.497	25.02	0.497 ± 0.0030	25.02 ± 0.1500	0.5995	2.663	25.00	2.663 ± 0.0055	25.00 ± 0.0551	0.2204
	0.500	25.17				2.658	24.95			
	0.494	24.87				2.669	25.06			

*n=3

C) Repeatability:

Table 5.5 Repeatability of Levofloxacin

298.5 nm

No.	Drug conc. (ppm)	Response	Conc. (ppm)
1	15	1.591	14.93
2		1.592	14.94
3		1.589	14.91
4		1.591	14.93
5		1.587	14.89
6		1.594	14.96
Mean		1.587	14.89
S.D.		0.0024	0.0242
% R.S.D.		0.1526	0.1626

269.29 nm

No.	Drug conc. (µg/ml)	Response	Conc. (µg/ml)
1	15	0.296	14.90
2		0.297	14.95
3		0.296	14.80
4		0.295	14.85
5		0.295	14.85
6		0.296	14.90
Mean		0.294	14.80
S.D.		0.001049	0.05244
% R.S.D.		0.3567	0.3543

5.2.2.3 LOD & LOQ:

Table 5.6 LOD & LOQ of Levofloxacin

298.5 nm

Drug	LOD (ppm)	LOQ (ppm)
Levofloxacin	0.068269	0.206876

269.29 nm

Drug	LOD (ppm)	LOQ (ppm)
Levofloxacin	0.1420	0.4231

5.2.2.4 Robustness:

Table 5.7 Robustness of Levofloxacin

298.5 nm

Conc.(µg/mL)	Absorbance		Conc. (µg/ml)	
	296.5	300.5	296.5	300.5
15	1.571	1.569	14.74	14.72
	1.573	1.568	14.76	14.72
	1.570	1.572	14.73	14.75
Average	1.570	1.568	14.73	14.72
S.D.	0.0015	0.0020	0.0152	0.01732
R.S.D	0.0972	0.1327	0.01037	0.1176
%Assay ± SD (n=3)			98.20 ± 0.1026	98.13 ± 0.1154

269.29 nm

Conc.(µg/mL)	Absorbance		Conc. (µg/ml)	
	267.29	271.29	267.29	271.29
15	0.281	0.284	14.14	14.29
	0.283	0.283	14.24	14.24
	0.282	0.282	14.19	14.19
Average	0.281	0.282	14.14	14.19
S.D.	0.001	0.001	0.05	0.05
R.S.D	0.3558	0.3546	0.3536	0.3523
%Assay ± SD (n=3)			94.59 ± 0.3350	94.60 ± 0.33

5.2.2.5 Recovery:

Table 5.8 Recovery study of Levofloxacin

Level of Recovery	Sample Conc. (µg/ml)	Amount of Std. added (µg/ml)	Total amount (µg/ml)	Absorbance (298.5 nm)	Amount Recovered (µg/ml)	% Recovery	Mean % Recovery
80 %	7.5	4.5	12	1.278	11.99	99.92	99.7567
	7.5	4.5	12	1.277	11.98	99.83	
	7.5	4.5	12	1.273	11.94	99.52	
100 %	7.5	7.5	15	1.608	15.09	100.60	100.4667
	7.5	7.5	15	1.611	15.12	100.80	
	7.5	7.5	15	1.598	15.00	100.00	
120 %	7.5	10.5	18	1.928	18.10	100.56	100.1133
	7.5	10.5	18	1.921	18.03	100.17	
	7.5	10.5	18	1.910	17.93	99.61	

Table 5.9 Summary of Validation parameters of Levofloxacin

PARAMETERS	RESULTS	
Wavelength	298.5 nm	269.29 nm
Linearity	5—25 µg/mL	
Equation	y = 0.1097x - 0.0092	y = 0.019x + 0.000
R^2	0.998	0.998
LOD (µg/mL)	0.0683	0.1420
LOQ (µg/mL)	0.2069	0.4231
Repeatability (% R.S.D., n=6)	0.1626	0.3543
Intraday Precision (% R.S.D., n=3)	0.4828	1.2415
Interday Precision (% R.S.D., n=3)	0.3766	1.3679
% Recovery	99.7 to 100.11 %	

5.2.3.1 Validation Parameters of Loteprednol etabonate:

Linearity:

Linearity range was found to be 5-25 µg/mL for Loteprednol etabonate in methanol with correlation co-efficient 0.999.

Table 5.10 Linearity of Loteprednol etabonate (5-25 µg/mL) at 269.29 nm

Conc. (ppm)	Mean Response ± S.D. *	% R.S.D.
5	0.1146 ± 0.0019	1.7148
10	0.2036 ± 0.0035	1.7196
15	0.2935 ± 0.0040	1.3755
20	0.3821 ± 0.0051	1.3588
25	0.4891 ± 0.0091	1.8620
Linearity Equation		y = 0.018x + 0.018
Correlation Coefficient		0.998
Slope		0.018
Intercept		0.018

* n= 6

Absorptivity: 202.992

Table 5.11 Linearity of Loteprednol etabonate (5-25 µg/mL) at 237.5 nm

Conc. (ppm)	Mean Response ± S.D. *	% R.S.D.
5	0.2531 ± 0.0028	1.1287
10	0.511 ± 0.0056	1.000
15	0.7376 ± 0.0128	1.7459
20	0.9603 ± 0.0177	1.8506
25	1.219 ± 0.0176	1.4518
Linearity Equation		y = 0.047x + 0.021
Correlation Coefficient		0.999
Slope		0.047
Intercept		0.021

* n= 6

Absorptivity: 495.3

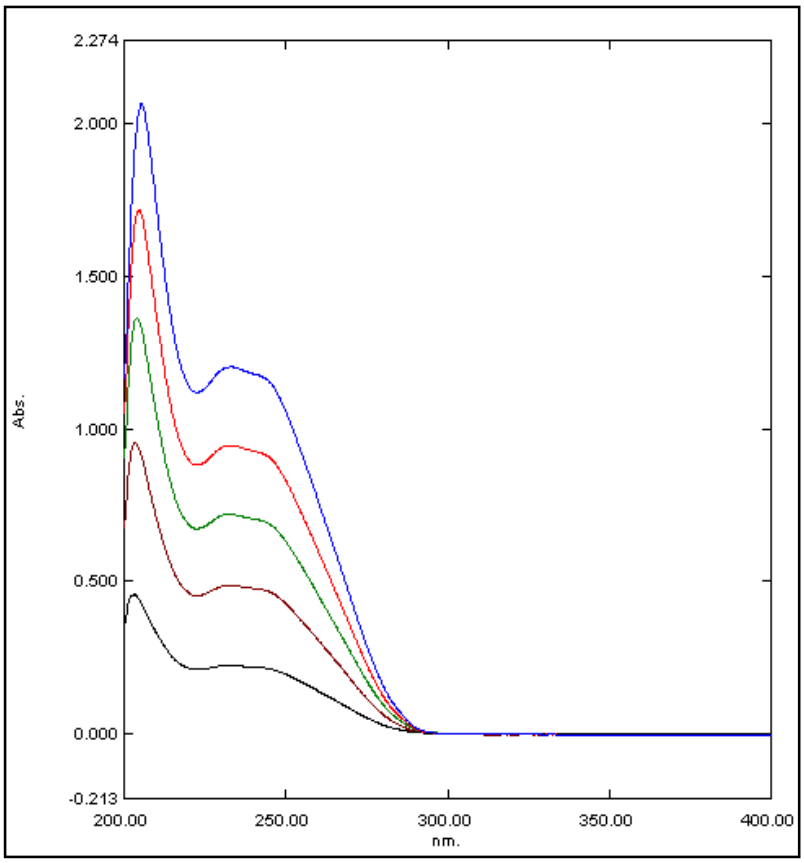

Fig. 5.5 Linearity Spectra for Loteprednol etabonate

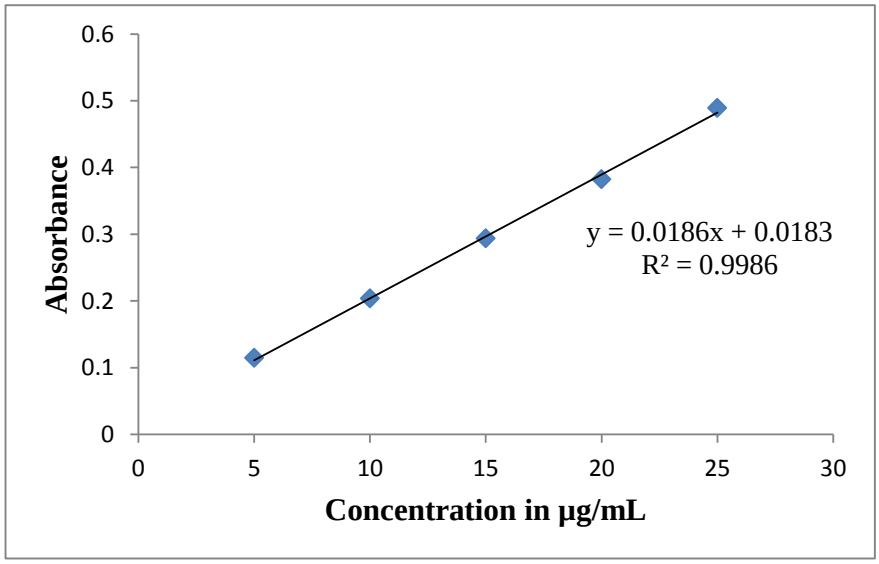

Fig. 5.6 Linearity Curve for Loteprednol etabonate at 269.29 nm

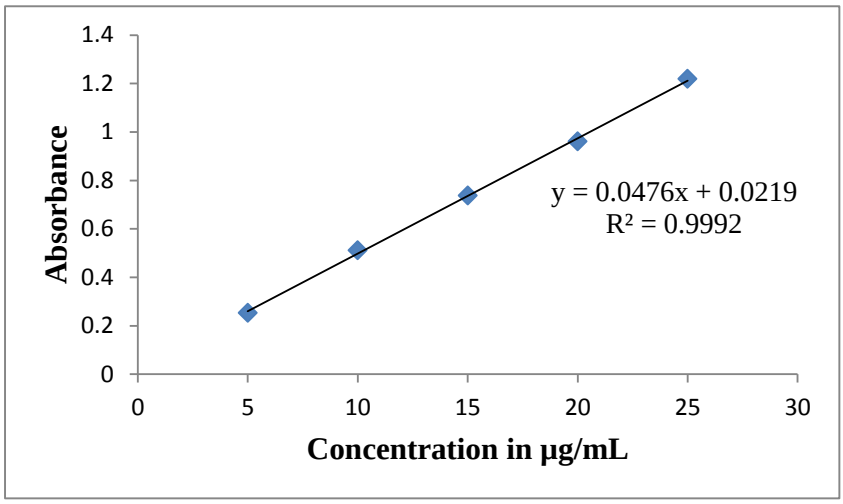

Fig. 5.7 Linearity Curve for Loteprednol etabonate at 237.5 nm

5.2.3.2 PRECISION:

A) Interday Precision:

Table 5.12 Interday Precision of Loteprednol etabonate at 269.29 nm

Conc. (ppm)	Abs.	Conc. (ppm)	Mean Abs. ± S.D.	Mean Conc. (ppm) ± S.D.	% R.S.D.
5	0.102	5.03	0.1017 ± 0.0015	5.01 ± 0.0721	1.4393
	0.100	4.93			
	0.103	5.07			
15	0.301	14.83	0.3043 ± 0.0035	14.99 ± 0.1704	1.1365
	0.304	14.98			
	0.308	15.17			
25	0.513	25.27	0.5063 ± 0.0070	24.94 ± 0.3465	1.3889
	0.499	24.58			
	0.507	24.98			

B) Intraday Precision:

Table 5.13 Intraday Precision of Loteprednol at 269.29 nm

Conc. (ppm)	Abs.	Conc. (ppm)	Mean Abs. ± S.D.	Mean Conc. (ppm) ± S.D.	% R.S.D.
5	0.105	5.17	0.1046 ± 0.0015	5.1562 ± 0.0753	1.4594
	0.103	5.07			
	0.106	5.22			
15	0.307	15.12	0.3023 ± 0.0041	14.8939 ± 0.2051	1.3771
	0.299	14.73			
	0.301	14.83			
25	0.499	24.58	0.5047 ± 0.0055	24.8614 ± 0.2713	1.0913
	0.505	24.88			
	0.510	25.12			

*n=3

C) Repeatability:

Table 5.14 Repeatability of Loteprednol etabonate at 269.29 nm

No.	Drug conc. (ppm)	Response	Conc. (ppm)
1	15	0.305	15.94
2		0.299	15.61
3		0.297	15.5
4		0.301	15.72
5		0.294	15.33
6		0.297	15.50
Mean		0.2988	15.60
S.D.		0.0038	0.2111
% R.S.D.		1.277	1.3537

5.2.3.3 LOD & LOQ:

Table 5.15 LOD & LOQ of Loteprednol etabonate

269.29 nm

Drug	LOD (ppm)	LOQ (ppm)
Loteprednol etabonate	0.059	0.196

5.2.3.4 Robustness:

Table 5.16 Robustness of Loteprednol etabonate

269.29 nm

Conc.(µg/mL)	Absorbance		Conc. µg/mL	
	267.29	271.29	267.29	271.29
15	0.299	0.283	15.61	14.72
	0.301	0.282	15.72	14.66
	0.303	0.284	15.83	14.77
Average	0.301	0.283	15.72	14.71
S.D.	0.002	0.001	0.11	0.055
R.S.D	0.6644	0.353	0.699	0.374
%Assay ± SD (n=3)			104.79 ± 0.7350	98.10 ± 0.3655

5.2.3.5 Recovery:

Table 5.17 Recovery study of Loteprednol etabonate

Level of Recovery	Sample Conc. (µg/ml)	Amount of Std. added (µg/ml)	Total amount (µg/ml)	Absorbance		Amount Recovered (µg/ml)	% Recovery	Mean % Recovery
				269.29 nm	298.5 nm			
80 %	2.5	1.5	4	0.318	1.278	3.94	98.50	99.95
	2.5	1.5	4	0.319	1.277	3.99	99.75	
	2.5	1.5	4	0.321	1.277	4.06	101.60	
100 %	2.5	2.5	5	0.403	1.608	5.04	100.80	100.03
	2.5	2.5	5	0.401	1.611	4.96	99.20	
	2.5	2.5	5	0.400	1.598	5.00	100.10	
120 %	2.5	3.5	6	0.483	1.928	6.04	100.67	100.83
	2.5	3.5	6	0.481	1.921	6.05	100.83	
	2.5	3.5	6	0.479	1.910	6.06	101.00	

Table 5.18 Summary of Validation parameters of Loteprednol etabonate

PARAMETERS	RESULTS
Wavelength	269.29 nm
Linearity	5 –25 µg/mL
Equation	y = 0.018x + 0.018
R^2	0.999
LOD (µg/mL)	0.1620
LOQ (µg/mL)	0.4912
Repeatability (% R.S.D., n=6)	1.3537
Intraday Precision (% R.S.D., n=3)	1.3093
Interday Precision (% R.S.D., n=3)	1.3216
% Recovery	100.27 %

Table 5.19 Analysis of Laboratory made Synthetic mixture of Levofloxacin & Loteprednol etabonate

Drug	Label Claim (%w/v)	%Assay± S.D.*	%RSD
Loteprednol etabonate	0.5	100.15 ±0.2596	0.2592
Levofloxacin	1.5	100.62 ±1.031	1.025

*n=3

5.2.4 DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN AND LOTEPREDNOL ETABONATE IN SYNTHETIC MIXTURE.

5.2.4.1 Instrument

- JASCO HPLC system; JASCO-PU-2080 plus-intelligent HPLC Pump; JASCO MD-2015 Plus multiwavelength detector, column ACE 5µ $C_{8,}$ 250×4.6 mm
- Analytical Balance Model: CX -220, Citizen
- Vacuum Pump Model: Rocker 600, Rocker-oil less vacuum pump
- Sonicator Model: TRANS-O-SONIC; D-compect
- Digital pH meter, Analab scientific Instrumentation Pvt. Ltd.
- Water bath Model: Water bath with digital temperature controller, EIE Instrument Pvt. Ltd.

5.2.4.2 Reagent and material

- API Levofloxacin gifted sample from Torrent Pharmaceuticals Pvt. Ltd.
- API Loteprednol etabonate gifted sample from Ajanta Pvt. Ltd.
- Methanol (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Methanol (HPLC Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Acetonitrile (HPLC Grade, Merck Apecialities Pvt. Ltd. Worli, Mumbai, India)
- Ammonium Acetate (anhydrous) (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Glacial Acetic acid (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Triethyl amine (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)

5.3.4 Method

5.3.4.1 Preparation of solutions

5.3.4.1.1 Preparation of standard stock solution of Levofloxacin and Loteprednol etabonate

Levofloxacin (25 mg) and Loteprednol etabonate (25mg) were accurately weighed and transferred to two separate 25 mL volumetric flasks. Dissolved in 10 mL methanol, sonicated

for 2 min and volume made up to mark with methanol to obtain standard stock solution having concentration 1000 µg/mL each.

From stock solution of Levofloxacin aliquots of 0.75, 1.50, 2.25, 3 & 3.75 ml were taken in different 10 ml Ambered color flask and diluted upto mark with mixture of 50:50 mixture of water and acetonitrile. So the resultant concentrations were 75, 150, 225, 300 and 375 µg/ml of Levofloxacin.

From stock solution of Loteprednol etabonate aliquots of 0.75, 1.50, 2.25, 3 & 3.75 ml in different 10 ml Ambered color flask were taken and diluted upto mark with mixture of 50:50 mixture of water and acetonitrile. So the resultant concentrations were 75, 150, 225, 300 and 375 µg/ml of Loteprednol etabonate.

5.3.4.1.2 Preparation of sample solution

Eye-drops of combination of Levofloxacin & Loteprednol etabonate has concentration of 0.5% and 1.5% respectively. Take aliquot of 0.2 mL in 10 mL of Ambered color flask and dilute upto mark with mixture of 50:50 mixture of water and acetonitrile. So the resultant concentration will be 300 µg/mL of Levofloxacin and 150 µg/mL of Loteprednol etabonate.

5.3.4.1.3 Mobile phase preparation:

Ammonium acetate buffer (20mM) with 3.1 pH:

Weighed accurately 770.8 mg of ammonium acetate and transferred in 500 mL of beaker. 500 mL of Millipore water was added and 0.5 mL of Tri-ethyl amine was added. pH was adjusted to 3.1 with glacial acetic acid. Then resulting solution was filtered through 0.22 µ Nylon filter. It was sonicated for 15 minute.

Acetonitrile: HPLC grade acetonitrile was used and it was sonicated for 15 minute.

5.3.4.2 OPTIMIZATION OF EXPERIMENTAL CONDITIONS

- Column: Zorbax C_8 column (250 mm ×4.6 mm i.d., 5µm)
- Mobile phase: Acetonitrile : ammonium acetate buffer (20 mM) added 0.5 mL TEA/500 ML adjusted to pH-3.1 by Glacial acetic acid

Table 5.20 Optimized and applied Gradient system for separation.

Time (minute)	Buffer	Acetonitrile
0	75	25
10	75	25
25	20	80
30	75	25
35	75	25

- Flow rate : 1.0 ml/min
- Injection volume : 20 µL
- Detection : 269 nm

5.3.4.3 Selection of detection wavelength:

The standard solution Levofloxacin and Loteprednol etabonate (10 µg/ml) was scanned over the range of 200 nm to 400 nm wavelengths. Both drug showed highest absorbance point at 269 nm. So the wavelength selected for the determination of Levofloxacin and Loteprednol etabonate at 269 nm.

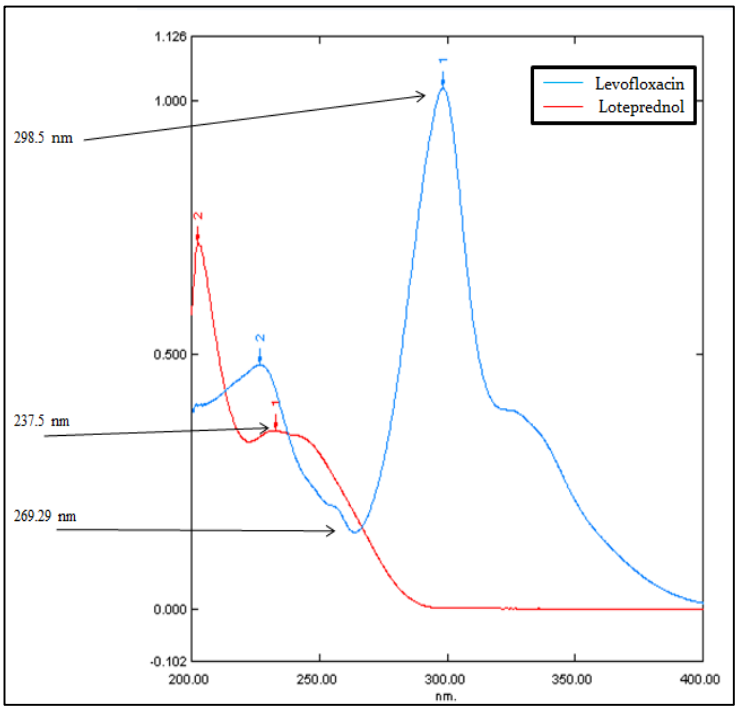

Fig 5.8 Overlay spectra of 10 µg/mL Levofloxacin & Loteprednol etabonate in methanol

5.3.4.3 Selection of Column:

Zorbax C_8 column (250 mm × 4.6 mm i.d., 5µm) was selected because with C_{18} column the separation time for both the peaks was higher resulting into more run time.

5.3.4.4 Selection of mobile phase:

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks on the chromatogram detect all the compounds. By slight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time. The mobile phase was selected on the basis of best separation, peak purity index, peak symmetry, theoretical plate etc. After of trial ammonium acetate buffer (pH 3.1): Acetonitrile Gradient scheme was selected.

Selection of Flow Rate

1 ml/minute tried first and it showed proper result so the flow rate 1 ml/minute selected for determination of Levofloxacin and Loteprednol etabonate.

5.3.5 Method Validation:

Validation Approach:

Validation of analytical method shall be done to establish by laboratory studies, that the performance of the method meet the requirement for the intended analytical application

5.3.5.1 Linearity and range:

Procedure:

The linearity was determined at five levels over the range of 75 to 375 ppm of concentration for Levofloxacin and 25 to 125 ppm of concentration for Loteprednol etabonate.

Solvent mixture was injected as blank, then standard solution as per test procedure and linearity solution preparations at each level in duplicate. Mean area was calculated at each

level and plotted a graph of mean area (y-axis) versus concentration in % (x-axis). Value of correlation co efficient (r), y-intercept, slope of regression line and residual sum of squares were calculated and recorded.

Table 5.21: Linearity of both Levofloxacin and Loteprednol etabonate

Sr. No.	Levofloxacin			Loteprednol etabonate		
	Con. (µg/ml)	Mean area ± S.D*	%RSD	Con. (µg/ml)	Mean area ± S.D*	%RSD
1	75	4466561 ± 73012	1.6346	25	891743 ± 8890	0.9969
2	150	7505841 ± 108272	1.4425	50	2080698 ± 20146	0.9682
3	225	10036914 ± 38456	1.3794	75	2900230 ± 27666	0.9539
4	300	12693389 ± 128656	1.0135	100	3975661 ± 37355	0.9395
5	375	15644066 ± 187137	1.1962	125	5129652 ± 43789	0.8536

*Indicates (n=3)

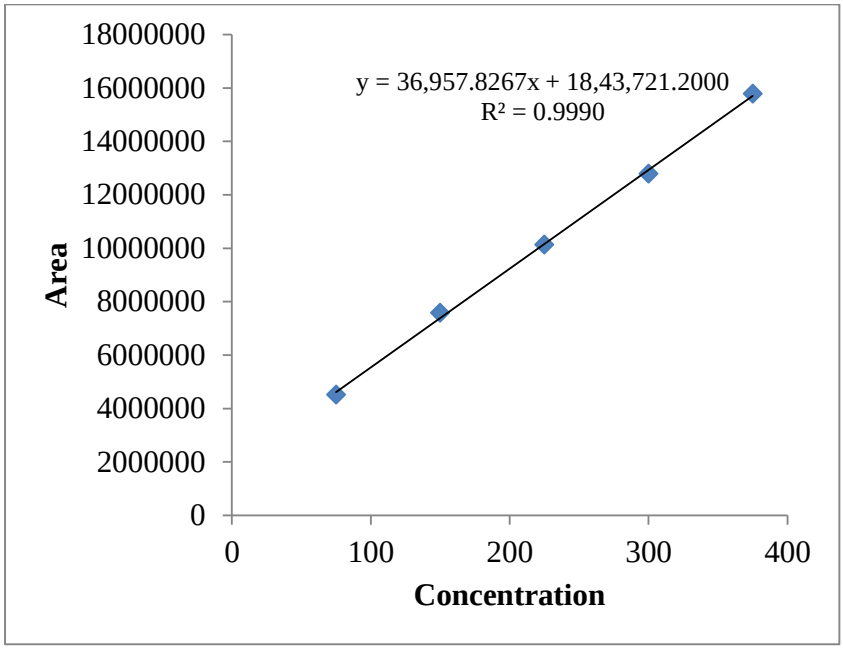

Figure 5.9: Linearity of Levofloxacin: 75 µg/mL to 375 µg/mL

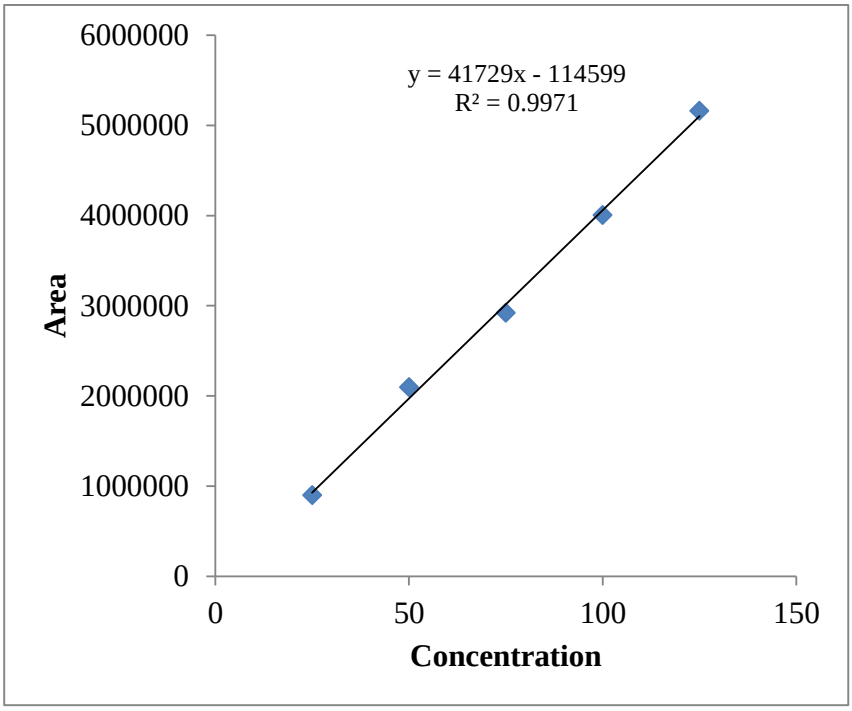

Figure 5.10: Linearity of Levofloxacin: 25 µg/mL to 125 µg/mL

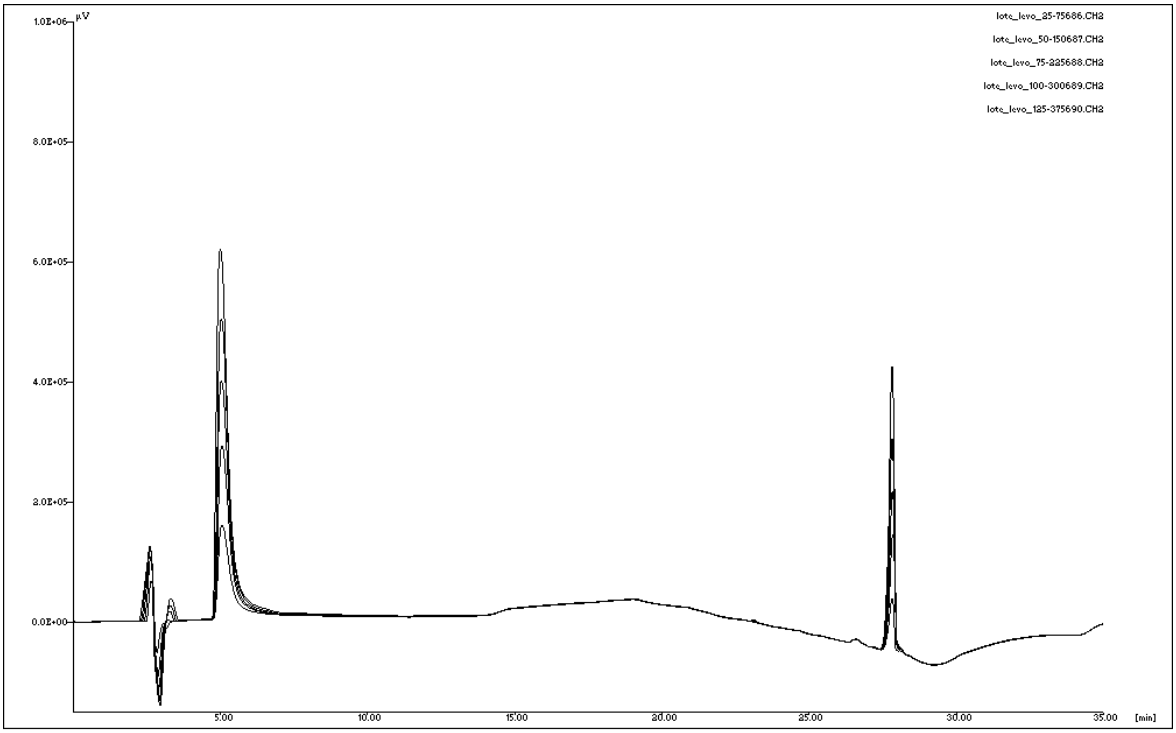

Fig 5.11 Linearity Overlay for both Levofloxacin (RT 4.9 min)
Loteprednol etabonate (RT 27.7 min)

5.3.5.2 Accuracy:

Accuracy of the measurement of Levofloxacin and Loteprednol etabonate was determined by standard addition method. Standard addition was done at three levels, 80%, 100% and 120% of a concentration in the linearity range.

Table 5.22 Accuracy data of Levofloxacin & Loteprednol etabonate by RP-HPLC

Drug	Initial conc. (µg/ml)	Std. solution added (µg/ml)	Final Conc. (µg/ml)	Accuracy			
				Conc. Recovered mean ± SD*	% RSD	% recovery mean ± SD*	% RSD
Levofloxacin	50	40	90	89.95 ± 0.3879	0.4313	99.94 ± 0.4315	0.4317
		50	100	99.49 ± 0.5961	0.5991	99.49 ± 0.5961	0.5991
		60	110	109.23 ± 0.7607	0.6965	99.30 ± 0.7008	0.7057

Loteprednol etabonate	50	40	90	89.48 ± 0.5311	0.5935	99.60 ± 0.3674	0.3689
		50	100	99.13 ± 0.7697	0.7764	99.13 ± 0.7697	0.7764
		60	110	109.35 ± 1.028	0.9404	99.41 ± 0.9361	0.9417

* indicates n=3

5.3.5.3 Repeatability

Table 5.23 Repeatability of Levofloxacin

No.	Conc.	Area	Recovered Conc.
1	300	12779846	295.91
2		12818791	296.96
3		12785269	296.05
4		12854180	297.91
5		12873073	298.43
6		12709521	294.11
	Average	12803446.67	296.5617
	SD*	58934.87	1.559775
	RSD	0.460305	0.5259

Table 5.24 Repeatability of Loteprednol etabonate

No.	Conc.	Area	Recovered Conc.
1	100	4099786	100.98
2		4089088	100.07
3		4102075	101.04
4		3989247	98.34
5		3987894	98.31
6		4016578	99.04
	Average	4047445	99.63
	SD*	55397.21	1.245697
	RSD	1.368696	1.2503

5.3.5.4 Intraday and interday precision

Table 5.25 Intraday precision data of Levofloxacin

Sr. No.	Concentration (µg/mL)	Peak area	
		Mean ± SD*	% RSD
1	75	4615139 ± 7586.09	0.2734
2	225	10114643 ± 21611.30	0.2592
3	375	15702924 ± 26688.38	0.1932

* indicates (n=3)

Table 5.26 Intraday precision data of Loteprednol etabonate

Sr. No.	Concentration (µg/mL)	Peak area	
		Mean ± SD*	% RSD
1	25	892748.66 ± 2394.01	0.2427
2	75	3012966.66 ± 13211.01	0.4266
3	125	5100020 ± 17897.78	0.3463

* Indicates (n=3)

Table 5.27 Interday precision data of Levofloxacin

Sr. No.	Concentration (µg/mL)	Peak area	
		Mean ± SD*	% RSD
1	75	4608982 ± 28292.34	1.018
2	225	10139817 ± 47696.96	0.5215
3	375	15703290 ± 83862.11	0.4523

* Indicates (n=3)

Table 5.28 Interday precision data of Loteprednol etabonate

Sr. No.	Concentration (µg/mL)	Peak area	
		Mean ± SD*	% RSD
1	25	891628.33 ± 6289.66	0.6379
2	75	2990293 ± 17769.35	0.5629
3	125	5115922 ± 39042.38	0.7460

* Indicates (n=3)

5.3.5.5 Robustness

Table 5.29 Robustness study of Levofloxacin

Parameter	Change	Mean ± % RSD	% Assay
pH (± 0.05)	3.15	12770821 ± 1.21	98.55
	3.05	12793865 ± 0.92	98.76
Wave Length (± 2nm)	259	12626172 ± 1.53	97.25
	263	12660548 ± 1.74	97.56

Table 5.30 Robustness study of Loteprednol etabonate

Parameter	Change	Mean ± % RSD	% Assay
pH (± 0.05)	3.15	4001966 ± 1.24	98.65
	3.05	4006974 ± 1.36	98.77
Wave Length (± 2nm)	259	4138420 ± 0.95	101.92
	263	4085007 ± 0.87	101.64

5.3.5.6 System suitability parameters

Table 5.31 System suitability parameters of the method

Sr. No.	**Parameters**	**Levofloxacin**		**Loteprednol etabonate**	
		Avg. ± SD*	**% RSD**	**Avg. ± SD***	**% RSD**
1	Retention time	4.9 ± 0.0011	0.0235	27.7 ± 0.0115	0.0416
2	Peak area	12693389 ± 128656.7	1.01357	3975661 ± 37355.04	0.9395
3	Tailing Factor	1.61 ± 0.01	0.6211	0.83 ± 0.0081	0.9820
4	Theoretical plates	1697 ± 27.50	1.72	198368 ± 119.26	0.061
5	Resolution	-		53.44	

* Indicates (n=3)

5.3.5.7 Application to Laboratory made synthetic mixture

The proposed validated method was successfully applied to the simultaneous determination of Levofloxacin & Loteprednol etabonate in Eye drops.

Table 5.32 Analysis of Levofloxacin & Loteprednol etabonate in formulations by proposed method

Drug	Labelled amount (% w/v)	Amount found		% Assay	
		Avg ± SD*	% RSD	Avg ± SD*	% RSD
Levofloxacin	1.5	1.519 ± 0.009	0.6324	101.26 ± 0.6110	0.6033
Loteprednol	0.5	0.517 ± 0.007	1.3983	103.46 ± 1.446	1.3983

* Indicates (n=3)

5.3.5.8 Summary of Validation Parameters

Table 5.33 Summary of Validation parameters

Sr. No.	**Parameters**	**Drug**	
		Levofloxacin	**Loteprednol etabonate**
1	Linearity range (µg/ml)	75-375	25-125
2	Regression equation	y = 36,957.8267x + 1,843,721	y = 41729x - 114599
3	Correlation coefficient (r^2)	0.999	0.997
4	Precision Intraday % RSD (n = 3) Interday % RSD (n = 3)	 0.2592 0.5215	 0.4266 0.5629
5	Repeatability	0.5259	1.2503
6	LOD (µg/ml)	9.659	29.27
7	LOQ (µg/ml)	1.167	3.536
8	% Recovery	0.5991	0.7764

5.3.5.9 Conclusion

By the virtue of the developed method, it can be concluded that reverse phase high performance liquid chromatography method is a reliable technique for the analysis of

combined formulations of Levofloxacin and Loteprednol etabonate; developed method is simple sensitive and specific which renders it suitable for routine analysis of Levofloxacin and Loteprednol etabonate from its combined dosage form. A good % recovery for both the drugs shows that the developed method is free of the interference of excipients used in the formulation.

CHAPTER 6

FUTURE SCOPE

6.1 FUTURE SCOPE

- Simultaneous estimation UV-Visible Spectrophotometric method for simultaneous estimation of Levofloxacin and Loteprednol in their combined dosage form can be developed.
- HPTLC (High performance thin layer chromatography) method for simultaneous estimation of Levofloxacin and Loteprednol in their combined dosage form can be developed.
- SFC (Super critical fluid chromatography) method for simultaneous estimation of Levofloxacin and Loteprednol in their combined dosage form can be developed.
- Raman spectrophotometry method for simultaneous estimation of Levofloxacin and Loteprednol in their combined dosage form can be developed.

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CHAPTER 7

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