ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF MOXIFLOXACIN AND TOLFENAMIC ACID IN BULK DRUG AND PHARMACEUTICAL DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT:

For the simultaneous measurement of tolfenamic acid and doxifloxacin in pharmaceutical formulations, the reverse phase HPLC technique was used to design and validate analytical methods for both drugs in bulk and pharmaceutical dose forms. The Agilent Tech. Gradient System with Auto Injector (DAD) & Gradient Detector was used to analyze the medication. equipped with a UV730D Absorbance detector, a Reverse Phase (Agilent) C18 column (4.6 x 250 mm; 5 µm), and Chemstation 10.1 software. The mobile phase consists of 30:70 v/v methanol and 0.05% acetic acid (pH 4.2 adjusted with triethylamine) at a flow rate of 1.0 mL/min. A UV detector was used for detection at 287 nm. Moxifloxacin and tolfenamic acid had respective retention periods of 5.237 and 3.762 minutes. With correlation values of 0.999 for both analytes, the technique showed a linear response spanning concentration ranges of 7.5-37.5 µg/mL for tolfenamic acid and 15-75 µg/mL for xifloxacin. The new method's specificity, linearity, accuracy, precision, robustness, and system adaptability were all validated in accordance with ICH recommendations. Recovery trials were used to determine the method's accuracy; mean recoveries for tolfenamic acid and doxifloxacin ranged from 98.23% to 100.51% and 99.88% to 101.75%, respectively.

KEYWORDS: *-RP-HPLC; Tolfenamic Acid; Moxifloxacin; Chromatography; Analytical Method; Validation; Bulk Drug; Method Development; Pharmaceutical Dosage Form; Simultaneous Estimation*

INTRODUCTION TO ANALYTICAL CHEMISTRY:

Analytical chemistry is the analysis of material samples to gain an understanding their chemical composition and structure. [1]

Analytical Chemistry is a measurement of science consisting of a collection of significant ideas and procedures applicable to all sectors of research and medicine. It seeks ever-improving methods of evaluating the chemical content of materials that are both natural and synthetic. In recent years, research in analytical chemistry has been primarily focused with the development and implementation of physicochemical & physical analytical methods, instrumental analysis, in which their speed and sensitivity have considerably beyond the classical methods of gravimetric and volumetric analysis. [2]

All researchers have benefited from analytical approaches. Analytical procedures have been enhanced as analytical tools based on physical measurement have advanced, assisted by electronics. Many Nobel Prizes in chemistry and physics have been awarded for the development of innovative analytical measurement techniques. A qualitative method offers information about the identity of atomic or molecular species or functional groups in the sample, whereas a quantitative method provides numerical information about the relative amount of one or more of these components. [3]

Currently instrumentation analysis focuses on comparing the data from the sample to that of the standard. Analytical chemistry encompasses chemical analysis, separation chemistry and instrumental analysis, including analytical methods and techniques. [4]

1.1.1 Classification of analytical methods:

- Analytical chemistry comprises of both qualitative and quantitative analysis.
- Qualitative analysis identifies elements, ions, and compounds in samples.
- Quantitative analysis determines the quantity of a given ingredient.

Qualitative analysis is subdivided in to following classical methods (Fig.No.1). [5]

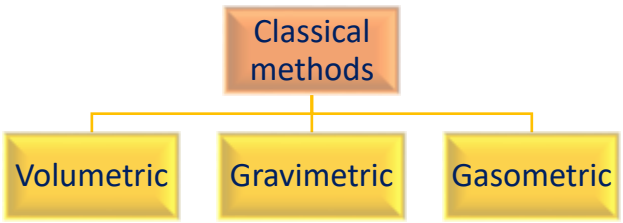


Fig.No.1 Flow chart of classification of classical methods.

A) Classical methods:

In the early days of chemistry, most analyses were carried out by traditional procedures by separating components of interest in a sample via precipitation, extraction, or distillation.

a) Volumetric methods:

It is also known as the titrimetric method and is preferred over gravimetry since it is faster and more convenient

a) Neutralization titration b) Non-aqueous titration c) Precipitation titration
d) Complexometric titration e) Redox titration

b) Gravimetric methods Gravimetric analysis is quantitative analysis by weight and is process of isolating and weighing the compound of known composition.

c) Gasometric method:

This method involves measurement of volume of gas by gas burettes or nitrometers. They measure the volume of the gas liberated in the given chemical reaction and volume decreased of gas in present of adsorbing agent under standard condition of temperature and pressure.

Quantitative Analysis is further classified into:

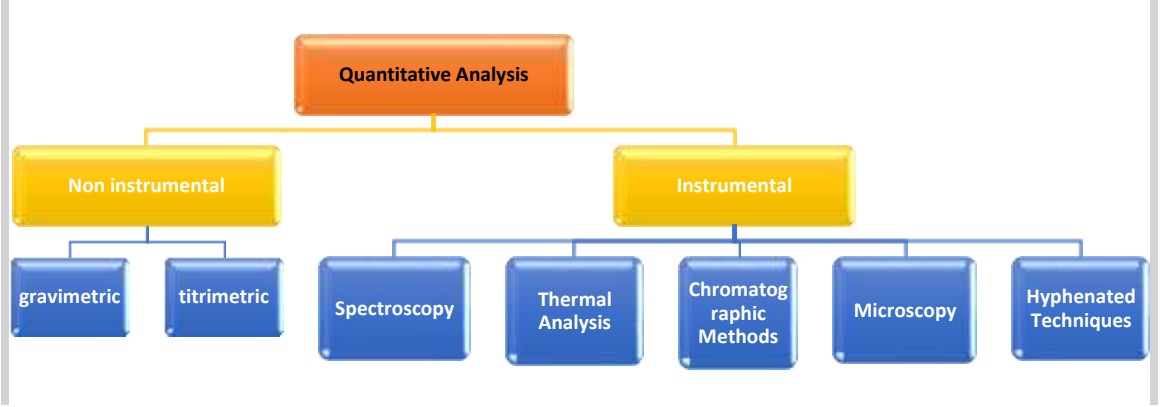


Fig.No.2: Flow chart of classification of quantitative analysis. [6]

➢ **B) INSTRUMENTAL METHODS OF CHEMICAL ANALYSIS**

Instrumental method is an exciting and fascinating part of chemical analysis that interacts with all areas of chemistry and with many other areas of pure and applied sciences (7, 8). Analytical Instruments plays an important role in the production and evaluation of new products.

1. Spectroscopy

- UV spectrophotometry
- Phosphorescence, Fluorescence

- Atomic spectrometry (emission and absorption)
- Infrared
- Raman
- X-ray
- Radiochemical technique including activation analysis
- Nuclear magnetic resonance spectroscopy
- Mass spectrometry
- Electron Spin Resonance spectroscopy

2. Electrochemical techniques

- Potentiometry
- Voltametry
- Amperometric technique
- Coulometry
- Electrogravimetry
- Conductometry technique

3. Chromatographic techniques

- GC
- HPLC
- HPTLC
- Electrophoresis
- Supercritical fluid chromatography (SFC)
- Ultra pressure liquid chromatography (UPLC)

4. Miscellaneous techniques

- Thermal analysis
- Kinetic technique

5. Microscopy

- Optical microscopy
- Electron microscopy
- Scanning probe microscopy

6. Hyphenated or combined techniques

- GC-MS (Gas Chromatography- Mass Spectrometry)
- LC-MS (Liquid Chromatography- Mass Spectrometry)
- ICP-MS (Inductively coupled Plasma Mass Spectrometry)
- GC-IR (Gas Chromatography-Infrared Spectroscopy)

1.2 Introduction to HPLC

1.2.1 Chromatography:

Nearly all the samples presented to the pharmaceutical analyst are mixtures, and sometime vary complex ones. The interfering substance can respond quantitatively to the analytical method for the desired component. Common example is the interference observed in absorption spectroscopy when two solutes have overlapping absorption bands. Sometimes the interference is a partial, gives non-quantitative response to the assay. [11]

➢ CLASSIFICATION OF CHROMATOGRAPHIC TECHNIQUES

All chromatographic separations are carried using a mobile and a stationary phase. As a result of this prerequisite, the primary classification of chromatography is based on the physical nature of the mobile phase. [12] Thus, all separation processes that utilize a gas as the mobile phase are classed as gas chromatography. Conversely, all separation processes that utilize a liquid as the mobile phase are classed as liquid chromatography. [13]

1.2.2 Definition of Chromatography:

Chromatography is unique in the history of analytical methodology and is probably the most powerful and versatile technique available to the modern analyst. In a single procedure it can separate a mixture into its individual components and simultaneously determine quantitatively the amount of each component present. [14]. It is based on the difference in the rate at which the components of mixture move through a porous medium (called stationary phase) under the influence of some solvent or gas (called moving phase).

The chromatographic method of separation, in general involve the following steps:

1. Adsorption or retention of a substance or substances on the stationary phase.
2. Separation of the adsorbed substance by the mobile phase.
3. Recovery of the separated substances by a continuous flow of the mobile phase; the method being called elution.
4. Quantitative and qualitative analysis of the eluted substances. [15]

1.2.3 HPLC:

HPLC is high resolution, high pressure and speed liquid chromatography. It has several times resolving power than open column liquid chromatography hence it is used for speedy resolution of complex mixture, determination & separation of species in a variety of organic, inorganic, and biological materials. [16]

1.2.4 Advantages of HPLC:

1. It offers a sensitive and precise approach for analysing complex material.

2. Ease of sample preparation and introduction.
3. Analysis is done quickly.
4. The analysis by hplc is specific, accurate and precise.
5. It offers advantage over gas chromatography in analysis of many polar, ionic substances, metabolic products and thermo labile as well as nonvolatile substances.

1.2.5 Instrumentation:

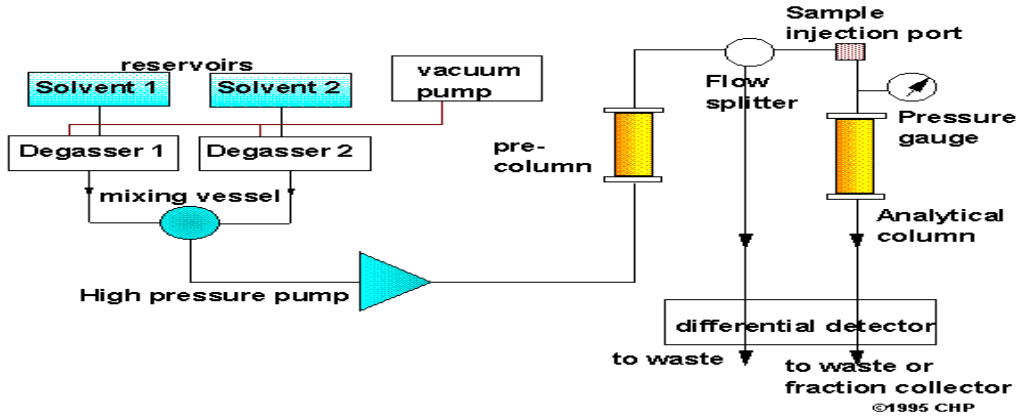


Fig no 3. Block diagram of HPLC.

1. Mobile phase reservoir and solvent treatment system:

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, each of which contain 500ml or more solvent. Reservoirs are commonly fitted with a way of eliminating dissolved gases, notably O2 and N2, which interfere by creating bubbles in the columns and detecting systems. [17]

Degassing:

Many liquids dissolve appreciable amounts of atmospheric gases, for example air, suspended air bubbles may be a major cause of practical problems in HPLC, specifically affecting the operation of the pump and the detector.. [18]

2. Pumps:

Pumps are most important component of HPLC and their performance directly affects the detector reproducibility and detector's sensitivity**.**

Their function is to force the liquid (mobile phase) through the column of finely packed particles.

The pumps are categorized as:

(a) Constant displacement pumps or Syringe pump
(b) Reciprocating pump
(c) Constant pressure or pneumatic pump. [19]

3. Sample injection system:

Three different modes of sample injection system that are used in HPLC are:

(a) Septum injectors:

They usually permit the introduction of the sample by a high pressure syringe through a self-sealing elastometer septum.

(b) Stop-flow septumless injection:

(c) The majority of the issues with septum injectors have been resolved. The flow of the mobile phase through the column is temporarily halted, and when the column achieves ambient pressure, the top of the column is opened, and the sample is injected at the top of the packing.

(d) Micro volume sampling valve:
These valves enable samples to be introduced reproducibly into pressurized columns without causing the least interruption of the mobile phase flow.

4. Columns:

a) Dimensions and Fittings: The dimensions of HPLC column are:
Material: stainless-steel (highly polished surface)
External diameter: 6.35 mm (or ≡ 0.25 inch)
Internal diameter: 4-5 mm (usual: 4.6 mm), and
Length: 10-30 cm (usual: 25 cm)

b) Fitting: Each end of the column is equipped with a stainless-steel gauze or frit with a mesh of 2 μm or less to keep the packing material, which typically has a particle diameter of 10, 5, or 3 μm.

A stainless-steel reducing union for a column of ID 4.6 mm type makes use of a ¼-$^{1}/_{6}$-inch union with a short length of 0.25 mm (or 0.01 inch) ID ptfe tube so as to connect the column to the detector. Porous plug of stainless steel or teflon are used in the end of the column to retain the packing material.

5. Detector:

a) Refractive Index Detector:

b) Ultra Violet Detectors:

c) Diode Array Detector (Multichannel Detector):

d) Fluorescence Detector:

e) Electrochemical Detectors:

1.2.6 Information about sample:

1. Number of components present in the sample.
2. PKa value of different components.
3. UV-spectra of each analyte.
4. Concentration range of each component.
5. Solubility behavior
6. Nature of sample (solid, liquid, semisolids).

At the beginning of method development one must consider these fact. The complete composition of the sample may not always be known since it may contain impurities, degradation products, and decomposition products. Before the sample is injected, we must reasonably sure that the detector selected will sense all components of interest. Variable wavelength detectors (UV) are the first choice because of wide applicability for most of the samples. For this, information on UV-spectra of the compounds can be separately measured or

obtained by photodiode-array (PDA) detector during separation. Other detectors are used when response to UV is poor. [21]

1.2.7 System suitability criteria's:

The System suitability parameters and recommendations are given in Table No.1 and Fig. No. 4.

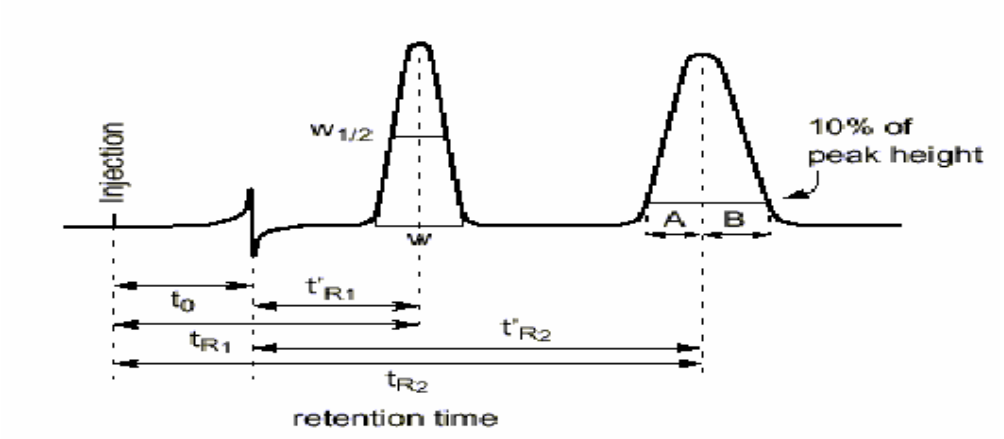


Fig. No.4. Ideal chromatogram for HPLC separation

Where: -

$w_{1/2}$ = peak width at half height

w = band width of the peak (intersection point of the inflection tangents with the zero line)

A = peak front at 10 % of peak height to peak maximum

B = peak maximum to peak end at 10 % of retention times

t_0 = dead time of a column (retention time of unretarded substance)

t_{R1}, t_{R2} …= retention time of components 1, 2…

t'_{R1}, t'_{R2}…= adjusted retention time of components 1, 2…

1. **Retention time:**

. The adjusted retention time (t'_{R1} or t'_{R2}) is the difference between total retention time and dead time. [22]

2. **Capacity factor (k'):**

It is the measure of the position of a sample peak in the chromatogram. It is specific for a given substance. k′ depends on the stationary phase, mobile phase, temperature, quality of the packing, etc. It should not be less than 1 and should not exceed 10 under selected chromatographic conditions

$$k' = \frac{t_{R1} - t_{R2}}{t_0}$$

3. **Relative retention (α)**

It is also known as separation factor and is the ratio between two capacity factors. The relative retention describes the ability of a chromatographic system to discriminate between two compounds. [23]

$$k'_2$$

$$\alpha = \frac{\quad}{k'_1}$$

4. Resolution (Rs):

The resolution Rs of a column provides a quantitative measure of its ability to separate two analytes. It is calculated from width and retention time of two peaks.

$$Rs = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

Where t_1 and t_2 are the retention time of first and second adjacent bands, $w_1$and w_2 are their baseline bandwidths.

Reliability of calculation is poor if Rs is < 2.0.

5. Number of theoretical plates (N):

This characterizes the quality of a column packing and mass transfer phenomenon. Larger the n, the more complicated sample mixtures can be separated with the column.

$$N = 16\left(\frac{t_{R_1}}{w}\right)^2 \quad \text{or} \quad N = 5.54\left(\frac{t_{R_1}}{w_{1/2}}\right)^2$$

The height equivalent to A TP h (HETP) is the length required to create chromatographic equilibrium between the mobile and stationary phases.
Because a high number of TP are required, 'h' should be as minimal as feasible. The value of 'h' is a criterion for the quality of a column; values depend on the particle size, the flow velocity, the mobile phase (viscosity) and especially on the quality of the packing.

$$h = \frac{L}{N}$$

L = Length of the column (cm)

N = Number of theoretical plates.

6. Asymmetry factor:

Symmetry is measured at 10 % of peak height. Ideally symmetry should be 1, i.e. a=b.

$$\text{Asymmetry} = b / a$$

Where, A is the distance from peak front to peak maximum and b is the distance from peak maximum to peak end. [23]

7. Tailing factor:

The accuracy of quantization decreases with increase in peak tailing. It should be $0.5 \leq T \leq 2$.

$$t = \frac{a+b}{2a}$$

Table No.1: System suitability parameters and recommendations [24]

1.3 Introduction to Spectrophotometry.

1.3.1 Spectroscopic method:

Spectroscopy is the branch of science dealing with the study of interaction of electromagnetic radiation with the matter. Spectroscopy is the process of experimentally measuring radiation frequencies emitted or absorbed and deducing energy levels from them. [25]

Spectroscopy is further divided into:

Atomic spectroscopy:

This concerns with the interaction of electromagnetic radiations with atoms, which are typically at their lowest energy state, known as the ground state.

Molecular spectroscopy:
This deals with the interaction of electromagnetic radiations with molecules. [26]

1.3.2 UV- visible absorption spectroscopy:

The basics of UV- visible spectrophotometry is the absorption of the UV- visible radiation, which causes the electronic transition within the molecules by the radiant energy of definite and narrow wavelength of monochromatic radiation.Light absorption in the UV-visible radiation causes the transition of an electron from a ground state and relaxation of energy takes place very rapidly. . [27]

Parameters	Recommendations
Capacity factor	The peak should be well resolved from other peaks and the void volume, generally 10 > k> 2
Repeatability	RSD Should be less than or equal to 2 %
Relative retention	Not essential so long as the resolution stated
Resolution (R_S)	Rs > 2 between the peak of interest and the closest eluting potential inteferent (impurity, excipient, degradation product, internal standard, etc.)
Tailing factor (T)	Value of T should be < 2
Theoretical Plates	Should be > 2000

1.4 According to the ICH guidelines published for the method validation following steps will be considered.

1. Accuracy.
2. Linearity.
3. Precision.
4. Repeatability.
5. Intermediate precision.
6. Detection limit.
7. Quantitation limit.
8. Specificity.
9. Range.
10. Robustness

2.DRUG PROFILE

TABLE no.2: DRUG PROFILE OF TOLFENAMIC ACID :-

Particular	Tolfenamic acid
Category	Nonsteroidal anti-inflammatory agent
Structure	
IUPAC Name	2-(3-chloro-2-methylanilino)benzoic acid
Molecular formula	$C_{14}H_{12}ClNO_2$
Molecular weight	261.704 g·mol−1
CAS No.	13710-19-5
Melting point	209° C to 214° C
Pka	3.7-4.3
Solubility	Methanol, ethanol, isopropanol
Half life	8.01-13.50 hours

Mechanism of Action:

Tolfenamic acid inhibits the biosynthesis of prostaglandins, and it also presents inhibitory actions on the prostaglandin receptors. As commonly thought, the mechanism of action of tolfenamic acid is based on the major mechanism of NSAIDs which consists of the

inhibition of COX-1 and COX-2 pathways to inhibit prostaglandin secretion and action and thus, to exert its anti-inflammatory and pain-blocking action.

Nonetheless, some report currently indicates that tolfenamic acid inhibits leukotriene B4 chemotaxis of human polymorphonuclear leukocytes leading to an inhibition of even 25% of the chemotactic response. This activity is a not ligand specific additional anti-inflammatory mechanism of tolfenamic acid.

Pharmacokinetics:

Absorption

Tolfenamic acid pharmacokinetic is marked by a short tmax of 0.94-2.04 h. It also presented a linear pharmacokinetic profile with an AUC from 13-50 mcg/ml.h if administered in a dose of 2-8 mg/kg respectively. The oral absorption is delayed and it gives a mean lag-time to absorption of 32 min. The peak plasma concentration of 11.1 mcg/ml. The bioavailability of tolfenamic acid is around 75%.

Route of Elimination

Tolfenamic acid is cleared relatively fast and it undergoes by hepatic metabolism where the produced metabolites are renally cleared as glucuronic acid conjugates. Most of the elimination occurs by extrarenal mechanisms in which the unchanged drug together with its glucuronide in urine accounts for only 8.8% of the administered dose.

Metabolism

The first pass metabolism accounts for 20% of the administered dose of tolfenamic acid. Urine metabolite studies have demonstrated the identification of five metabolites from which three of them are monohydroxylated, one is monohydroxylated and hydroxylated and one last metabolite that presented and oxidized methyl group to form a carboxyl group. Two of these hydroxylated metabolites are N-(2-hydroxymethyl-3-chlorophenyl)-anthranilic acid and N-(2-hydroxymethyl-3-chloro-4-hydroxyphenyl)-anthranilic acid.

Volume of distribution

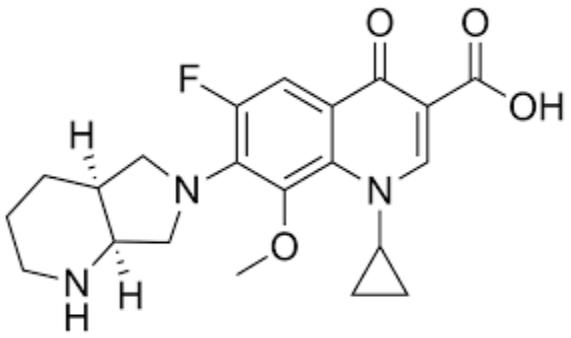
The volume of distribution is of 1.79-3.2 L/kg. When tested intravenously, the reported steady-state volume of distribution was 0.33 L/kg.

Side Effect

- Indigestion
- Dizziness
- Headache

- Drowsiness
- Stomach pain
- Vomiting
- Nausea
- Diarrhea

Table no.3 : DRUG PROFILE OF MOXIFLOXACIN : -

Particular	**Moxifloxacin**
Category	Antibiotic
Structure	
IUPAC Name	1-Cyclopropyl-7-[(1S,6S)-2,8-diazabicyclo[4.3.0]nonan-8-yl] - 6-fluoro-8-methoxy-4-oxoquinoline-3-carboxylic acid
Molecular formula	$C_{21}H_{24}FN_3O_4$
Molecular weight	401.431 g/mol
CAS No.	151096-09-2
Solubility	Water, methanol, ethanol, DMSO
Half life	8.2-15.1 hours

Mechanism of Action:

Tolfenamic acid inhibits the biosynthesis of prostaglandins, and it also presents inhibitory actions on the prostaglandin receptors. As commonly thought, the mechanism of action of tolfenamic acid is based on the major mechanism of NSAIDs which consists of the inhibition of COX-1 and COX-2 pathways to inhibit prostaglandin secretion and action and thus, to exert its anti-inflammatory and pain-blocking action.

Nonetheless, some report currently indicates that tolfenamic acid inhibits leukotriene B4 chemotaxis of human polymorphonuclear leukocytes leading to an inhibition of even 25%

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Volume of distribution

The volume of distribution is of 1.79-3.2 L/kg. When tested intravenously, the reported steady-state volume of distribution was 0.33 L/kg.

Side Effect

- Black, tarry stools.
- bleeding gums.
- blisters.
- bloating or swelling of the face, arms, hands, lower legs, or feet.
- blood in the urine or stools.
- blurred vision.
- bone pain.

3. MATERIAL AND INSTRUMENTS

3.1 Selection and Procurement of Drug

Drug sample supplier

Table 4: Drug and Drug Supplier

Name of Drug	Drug Supplier
Moxifloxacin	RSITC
Tolfenamic acid	RSITC

List of reagents & chemicals used

Table 5: List of Reagents and Chemicals used

Sr. No.	Name of chemicals	Manufacturer.
1.	Methanol (HPLC grade)	Merck Ltd., India
2.	Acetonitrile (HPLC grade)	Merck Ltd., India
3.	Ortho-phosphoric Acid buffer (HPLC grade)	Merck Ltd., India
4	Tri-ethyl amine	Merck Ltd., India

3.2 Selection of formulation:

Marketed Preparation**:**

Brand Name: **BOLUS**
Content: Moxifloxacin and Tolfenamic acid

The marketed preparation was obtained from local market and is referred here after in this thesis by the name as such.

3.3 Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Moxifloxacin and Tolfenamic acid.

Instruments:

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, (DAD) & Gradient Detector. Equipped with Reverse Phase (Agilent) C_{18} column (4.6mm x 250mm; 5µm), and UV730D Absorbance detector and running chemstation 10.1 software.

3.4 Instruments and Equipment's

Table. 6: Instrument (HPLC) Details used during Method Development

	Name of Instrument	Company Name
1	HPLC Instrument	Agilent Tech. Gradient System with Auto injector (Chemstation software)
2	UV-Spectrophotometer	Analytical Technology
3	Column(C_{18})	Agilent C_{18} (250mmX 4.6mm,5µm) :
4	pH meter	VSI pH meter(VSI 1-B)
5	Balance	WENSAR™ High Resolution Balance.
6	Sonicator	Ultrasonic's electronic instrument

4. EXPERIMENTAL WORK:

4.1 HPLC:

4.1.1 Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Moxifloxacin and Tolfenamic acid.

Instruments:

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, DAD Detector. Equiped with Reverse Phase C_{18} (Agilent) with 250 mm x4.6; (5µm), UV730D Absorbance detector and running chemstation 10.1 software.

Table 7: List of instruments

	Name of Instrument	Company Name
1	HPLC Instrument	Agilent Tech. Gradient System with Auto injector

2	UV-Spectrophotometer	Analytical Technologies Limited
3	Column(C_{18})	Agilent C_{18} (250mmX 4.6mm,5µm)
4	pH meter	VSI pH meter(VSI 1-B)
5	Balance	WENSAR™ High Resolution Balance.
6	Sonicator	Ultrasonics electronic instrument

a) Chromatographic conditions:

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

Table No.8: chromatographic conditions (HPLC) details used during method

Development

1.	HPLC	Agilent Tech. Gradient System with Auto injector
2.	Software	Chemstation
3.	Column	(Agilent) C18 column (4.6mm x 250mm)
4.	Particle size packing	5 µm
5.	Stationary phase	C-18 (Agilent)
6.	Mobile Phase	Methanol: Water (0.05% Acetic acid) PH adjusted 4.2 With TEA 30 : 70
7.	Detection Wavelength	287 nm
8.	Flow rate	1 ml/min
9.	Temperature	Ambient
10.	Sample size	20 µl
11.	pH	4.2
12.	Run Time	10 min
13.	Filter paper	0.45 µm

4.2 Study of Moxifloxacin and Tolfenamic acid on the chromatographic conditions used in method development of HPLC for the Following Mobile phase were tried:

4.2.1 METHOD DEVELOPMENT OF HPLC:

- **List of Mobile Phase:**
- **Table No.9: Selection of mobile Phase.**

Sr.No.	Mobile Phase
1.	Methanol :0.05% Acetic Acid (90:10 %v/v) 287 nm, 1 ml/min,150mmX 4.6-column
2	Methanol : Water 0.05% Acetic Acid (50 : 50%v/v),287 nm flow 0.7 ml/min,150 mmX 4.6
3	Methanol : water 0.05% Acetic Acid (90 : 10%v/v) PH3,287 nm, flow 0.7ml/min, 250 mmX 4.6
4	Methanol : 0.05% Acetic Acid (50 : 50%v/v),287 nm, flow 0.7ml/min, 250 mmX 4.6
5	Methanol : 0.05% Acetic Acid (30 : 70%v/v)287 nm, flow 0.7 ml/min, 250 mmX 4.6
6	Methanol : 0.05% Acetic Acid (30 : 70%v/v)287 nm, flow 0.7 ml/min, 250 mmX 4.6
7	Methanol: 0.05% acetic acid PH adjusted 4.2 with TEA (30:70% v/v) 287 nm,1 ml/min, 250 mmX 4.6

4.3. Analysis of standard drugs was done by following parameters:

- Melting point
- Solubility
- UV spectra and λ_{max}
- HPLC chromatogram and retention time

4.4. Selection of wavelength by UV-Visible Spectrophotometry: -

4.5.1. Preparation of standard stock solution:

- **Moxifloxacin standard stock solution: (Stock I)**
  An accurately weighed quantity, 15 mg of Moxifloxacin (MXF) was dissolved in Methanol in a 100 ml volumetric flask and volume made up to 100 ml to produce a solution of 1500 µg/ml.
- **Tolfenamic acid standard stock solution: (Stock II)**
  An accurately weighed quantity, 7.5 mg of Tolfenamic acid (TFM) was dissolved in Methanol in 100 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 750 µg/ml.
- **Preparation of Stock Standard Combination Solution :(Stock III) [MXF+TFM]**
  Accurately weight and transfer 15 mg Moxifloxacin and Tolfenamic acid 7.5 mg working standard into 100 ml volumetric flask as about diluent Methanol completely and make volume up to the mark with the same solvent to get 1500 µg/ml standard Moxifloxacin and 750 µg/ml for Tolfenamic acid (stock solution) and 15 min sonicate

to dissolve it and remove the unwanted gas, further an aliquots portion of Moxifloxacin and Tolfenamic acid stock solution in ratio of 30:70 were mixed in volumetric flask in 10 ml and volume was adjusted up to mark with mobile phase from the resulting solution 0.2 ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with Methanol: (0.05% Acetic acid)PH 4.2 With TEA, prepared in (3.0 ml Methanol: 7.0 ml 0.05% acetic acid)solvent .

4.5.2. HPLC used for chromatographic condition applies on the Preparation of standard solution: -

- **Preparation of std. Moxifloxacin solution: (Stock I)**
  From the freshly prepared standard stock solution (1500 µg/ml), 0.2 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration of 30 µg/ml.
- **Preparation of std. Tolfenamic acid solution: (Stock II)**
  From the freshly prepared standard stock solution (750 µg/ml), 0.2 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 15 µg/ml.
- **Preparation of std. Moxifloxacin and Tolfenamic acid solution :(Stock III)**
  From the freshly prepared standard stock solution (1500 µg/ml:750 µg/ml), 0.1 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 15 µg/ml of Moxifloxacin and 7.5 µg/ml was Tolfenamic acid.

4.5.3. Selection of mobile phase:

Each mobile phase was vacuum degassed and filtered through 0.45µ membrane filter. The mobile phase was allowed to equilibrate until steady baseline was obtained. From the various mobile phases tried, mobile phase containing Methanol & 0.05% Acetic Acid with pH adjust (4.2 with TEA) 0.05% Acetic Acid was selected since it gave sharp, well resolved peaks with symmetry within the limits and significant reproducible retention time for Moxifloxacin and Tolfenamic acid.

4.6. Studies of Calibration plot: -

4.6.1. Optimization of Chromatographic condition:

The following chromatographic conditions were established by trial and error and were kept constant throughout the analysis.

Column	: Agilent C18 (250 mm× 4.6mm)
Particle size packing	: 5µm
Detection wavelength	: 287 nm
Flow rate	: 1 ml/min
Temperature	: Ambient

Sample size : 20 µl
Mobile phase : Methanol: water (0.05% Acetic Acid) PH 4.2 with TEA (30 : 70)

4.7. Procedure for calibration curve of Moxifloxacin and Tolfenamic acid:

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. From the freshly prepared standard stock solution, pipette out 15 mg Moxifloxacin and 7.5 mg Tolfenamic acid in 100 ml of volumetric flask and diluted with mobile phase. From it 0.1, 0.2, 0.3, 0.4 and 0.5 ml of solution were pipette out in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 15,30,45,60,75 µg/ml of Moxifloxacin and 7.5,15,22.5,30,37.5 µg/ml of Tolfenamic acid.

Sample were injected and peaks were recorded at 287 nm as the graph plotted as concentration of drug verses peak area is depicted in respectively.

4.8. Study of system suitability parameters: The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from five replicate injections of standard solution.

4.9.1. Calibration Experiment:

❖ **RP-HPLC Method:**

a) Preparation of Calibration curve standard: The above standard stock solution (1500:750µg/ml) of Moxifloxacin and Tolfenamic acid was diluted with mobile phase to yield Five calibration curve (cc) standards with concentrations of 15,30,45,60,75 µg/ml of Moxifloxacin and 7.5,15,22.5,30,37.5 µg/ml of Tolfenamic acid

▪ **UV Spectrophotometric method:**

a) Selection of detection Wavelength:

Standard solutions were scanned in the range of 200-400nm, against 10 ml Methanol and volume make with Methanol solvent system as reference Moxifloxacin and Tolfenamic acid were showed absorbance maxima (lamda max) at 293 nm and 286 nm respectively **.**
If Two Moxifloxacin and Tolfenamic acid sample Interact with this point is called Isobestic point Then detection of wavelength in isobestic point in 287 nm were selection wavelength is HPLC Method can be used.

b) Calibration standard drug and regression equation data :

From the standard stock solution of Moxifloxacin and Tolfenamic acid, different concentration were prepared respectively in the range of 15-75 µg/ml for Moxifloxacin and 7.5-37.5 µg/ml

for Tolfenamic acid and measured at 293 nm and 286 nm. The calibration curves were plotted and Regression equation data presented in.

b) Calibration runs and regression analysis:

These calibration standard solutions were analyzed in three replicates using the under mentioned chromatographic conditions.

- Analytical column: Agilent C18 Column (100mm x 4.6mm, 2.5µm partical size).
- Injection volume : 20µl.
- Flow rate : 1 ml/min.
- Mobile phase : Methanol: 0.05% Acetic Acid 0.1% PH 4.2 with TEA (30: 70 % V/V).
- Detection : 287 nm.

4.10. Validation of method for analysis of Moxifloxacin and Tolfenamic acid:

➢ The developed method was validated as per ICH guidelines.

4.10.1 Linearity:

Linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

- **Determination:**
  The linearity of the analytical method is determined by mathematical treatment of test results obtained by analysis of samples with analyte concentrations across the claimed range. Area is plotted graphically as a function of analyte concentration Percentage curve fittings are calculated**.**
  Acceptance Criteria: The plot should be linear passing through the origin.
  Correlation Coefficient should not be less than 0.999. The Result are shown in;

- **Preparation of standard stock solution for linearity:**
  Weight 15 mg of Moxifloxacin and 7.5 mg of Tolfenamic acid were weighed and transfered to 100 mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. 0.1 ml of this solution diluted upto 10 ml volumetric flask with diluents was added to make up the volume.

- **Preparation of linearity solution:**
  A series of standard preparations of working standard of were prepared.

Table No.10: Table of linearity for Rp-HPLC Method

Concentration (µg/mL)	
Moxifloxacin	Tolfenamic acid
15	7.5
30	15
45	22.5
60	30
75	37.5

4.10.2 Accuracy (recovery):

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyte. The accuracy is calculated from the test results as the percentage of analyte recovered by the assay**.**

- **Acceptance Criteria:**
  Mean recovery should be in the range of 98-102%.
  The Relative Standard Deviation should not be more than 2.0%.

- **Preparation of standard stock solution:**
  15 mg of Moxifloxacin and 7.5 mg of Tolfenamic acid working standards were weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume 0.1 ml of this solution diluted upto 10 ml with diluent.

- **Application of proposed method for analysis of Tablet formulation:**
- **Accuracy**

The accuracy was determined by Moxifloxacin and Tolfenamic acid (equivalent to 15 mg of Moxifloxacin and 7.5 mg of Tolfenamic acid (80 %, 100 % and 120 % of the label claimed, respectively) to quantity equivalent to average weight of marketed tablets. This powder mixture containing 15 mg of Moxifloxacin and 7.5 mg of Tolfenamic acid were triturated and then subjected to chromatographic analysis using the described method. The resulting mixtures were analyzed in duplicates over two days. The % recovery of added drug was taken as a measure of accuracy**.** The Result are shown in; **.**

Sample	Amount Added (mg)	
	Moxifloxacin	Tolfenamic acid
Accuracy 80%	12	6
Accuracy 100%	15	7.5
Accuracy 120%	18	9

Table No. 11: Table of Accuracy Rp-HPLC Method

4.10.3 Repeatability:

Precision of the system was determined with the sample of RP-HPLC Method for. Two replicates of sample solution containing 15 mg of Moxifloxacin and 7.5 mg Tolfenamic acid were injected and peak areas were measured and % RSD was calculated. is was repeated for five times.

- **Application of proposed method for analysis of Repeatability:**

15 mg of Moxifloxacin and 7.5 mg Tolfenamic acid were weighed and transferred to 10 mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. The above solution was filtered through 0.45µm membrane filter 0.1 ml of this solution diluted upto 10 ml with diluent.

4.10.4 Precision:

Precision of an analytical method is the degree of agreement among Individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Also, the results obtained were subjected to one-way ANOVA and within-day mean square and between-day mean square was determined and compared using F-test.

- **Result of Intra day and Inter day Precision studies on RP-HPLC method for Moxifloxacin and Tolfenamic acid**

4.10.4.1 Intra-day precision:

Sample solutions containing 15 mg of Moxifloxacin and 7.5 mg three different concentration (7.5µg/ml,22.5µg/ml,37.5µg/ml) Tolfenamic acid and (15µg/ml,45µg/ml,75µg/ml) Moxifloxacin were analyzed three times on the same day and %R.S. D was calculated.

4.10.4.2 Inter-day precision:

Sample solutions containing 15 mg of Moxifloxacin and 7.5 mg three different concentration (7.5µg/ml,22.5µg/ml,37.5µg/ml) Tolfenamic acid and (15µg/ml,45µg/ml,75µg/ml) Moxifloxacin different days and % R.S.D was calculated. It is usually expressed as standard deviation or relative standard deviation.

Acceptance criteria:

The Relative Standard Deviation should not be more than 2% for test

- **Preparation of standard stock solution:**
  15 mg of Moxifloxacin and 7.5 mg Tolfenamic acid working standards were weighed and transfered to 10 mL volumetric flask & diluent was added to make up the volume. 0.1 ml of this solution diluted upto 10 ml with diluent.

4.10.5. Robustness:

The mobile phase composition was changed in (±1 ml/ min^{-1}**)** proportion and the flow rate was of methanol: 0.05% Acetic Acid 0.1% PH 4.2 with TEA (30:70) in the mobile phase composition (±1ml/ min^{-1}**)** and the change in detection wavelength (±1 ml/ min^{-1}**)** and the effect of the results were examined it was performed using 15 µg/ml and 30 µg/ml solution of Moxifloxacin and Tolfenamic acid in triplicate
Based on the S.D. of the response and the slope of calibration curve, the detection limit (DL) was calculated as,

$$DL = \frac{3.3\sigma}{S}$$

Where,
σ = the S.D. of the y-intercepts of regression lines.
S = the slope of the calibration curve.
The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

4.10.7 Quantitation Limit

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

$$QL = \frac{10\sigma}{S}$$

Where,
σ = the S.D. of the y-intercepts of regression lines.
S = the slope of the calibration curve.
The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

4.10.8 Analysis of marketed formulation.

To determine the content of Moxifloxacin and Tolfenamic acid in marketed tablets (label claim 15 mg of Moxifloxacin and 7.5 mg Tolfenamic acid), 20 tablets powder weighed in 2.50 gms. equivalent to weighed in 25 mg with 10 mL Methanol. To ensure complete extraction it was sonicated for 15 min. 0.5 mL of supernatant was then diluted up to 10 mL with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted
Regression equation was generated using peak areas of standard solutions. Using the regression equation and peak area of the sample the amount of Moxifloxacin and Tolfenamic acid in the sample was calculated.

5. RESULT:

5.1. Preliminary studies on Tolfenamic acid and Moxifloxacin.

5.1.1. Melting point

The procured reference standard of Tolfenamic acid and Moxifloxacin were found to melt in the range of 210-214^{0}C and 238-242^{0}C respectively.

5.1.2. Solubility

The drug was found to be

- Freely soluble in, methanol.
- Practically insoluble in water, but freely soluble in organic solvents.

5.1.3. UV Spectroscopy

UV absorption of 10 µg/mL solution of Tolfenamic acid and Moxifloxacin in methanol was generated and absorbance was taken in the range of 200-400 nm. λmax of Tolfenamic acid and Moxifloxacin in Methanol was found to be 286 nm and 293 nm respectively.

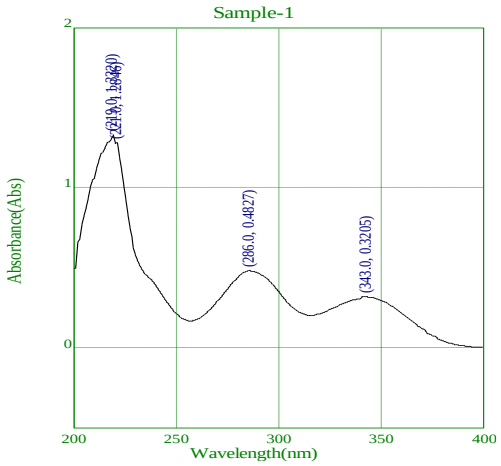


Fig No.5: UV Spectrum of Tolfenamic acid

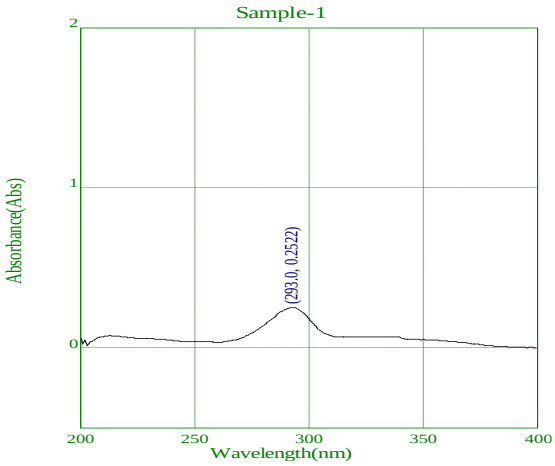


Fig No.6: UV Spectrum of Moxifloxacin

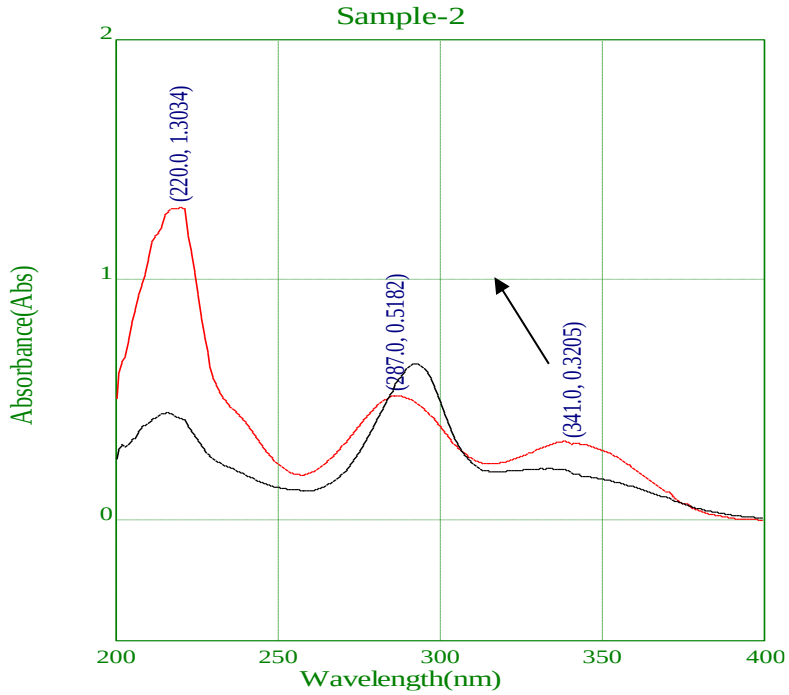


Fig.7: Iso-absorptive point of Tolfenamic acid and Moxifloxacin

5.1.4. Studies on the chromatographic behavior of Tolfenamic acid and Moxifloxacin

TABLE NO-12: Chromatographic behavior of Tolfenamic acid and Moxifloxacin mobile phase of various compositions.

Trial No.	Mobile Phase composition with flow rate	Observation	Result
1.	Methanol :0.05% Acetic Acid (90:10 %v/v) 287 nm, 1 ml/min,150mmX 4.6-column	Merge peak	Rejected
2	Methanol : Water 0.05% Acetic Acid (50 : 50%v/v),287 nm flow 0.7 ml/min,150 mmX 4.6	No Sharp peak(Merge peak)	Rejected
3	Methanol : water 0.05% Acetic Acid (90 : 10%v/v) PH3,287 nm, flow 0.7ml/min, 250 mmX 4.6	Merge peak	Rejected
4	Methanol : 0.05% Acetic Acid (50 : 50%v/v),287 nm, flow 0.7ml/min, 250 mmX 4.6	Resolve peak was not found	Rejected

5	Methanol : 0.05% Acetic Acid (30 : 70%v/v)287 nm, flow 0.7 ml/min, 250 mmX 4.6	Larger RT	Modified
6	Methanol : 0.05% Acetic Acid (30 : 70%v/v)287 nm, flow 0.7 ml/min, 250 mmX 4.6	Larger RT	Modified
7	Methanol: 0.05% acetic acid PH adjusted 4.2 with TEA (30:70% v/v) 287 nm,1 ml/min, 250 mmX 4.6	Sharp Peak obtain	Selected

Thus, from the above, it has been observed that, using mobile phase of Methanol + 0.05% acetic acid PH adjusted 4.2 with TEA (30+70% v/v) 287 nm,1ml, gave adequate retention time at 3.762 min and 5.237 min. with good peak shape (Theoretical plates of 5650 of Tolfenamic acid & 7356 of Moxifloxacin.

Chromatogram of Final Trial :

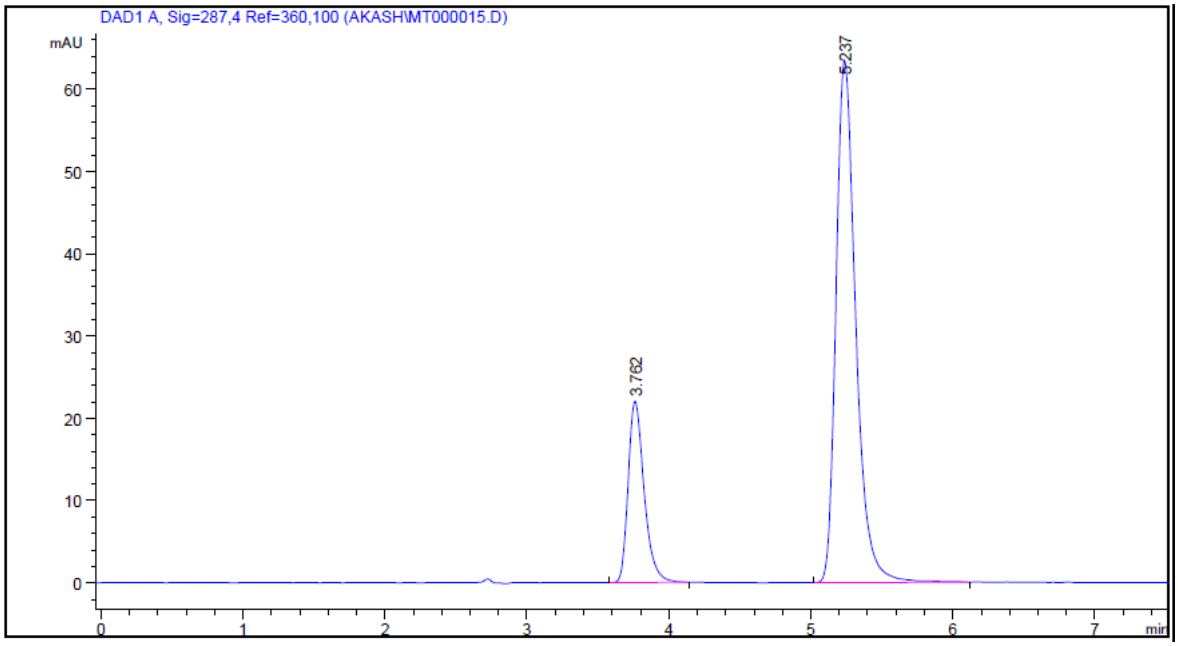


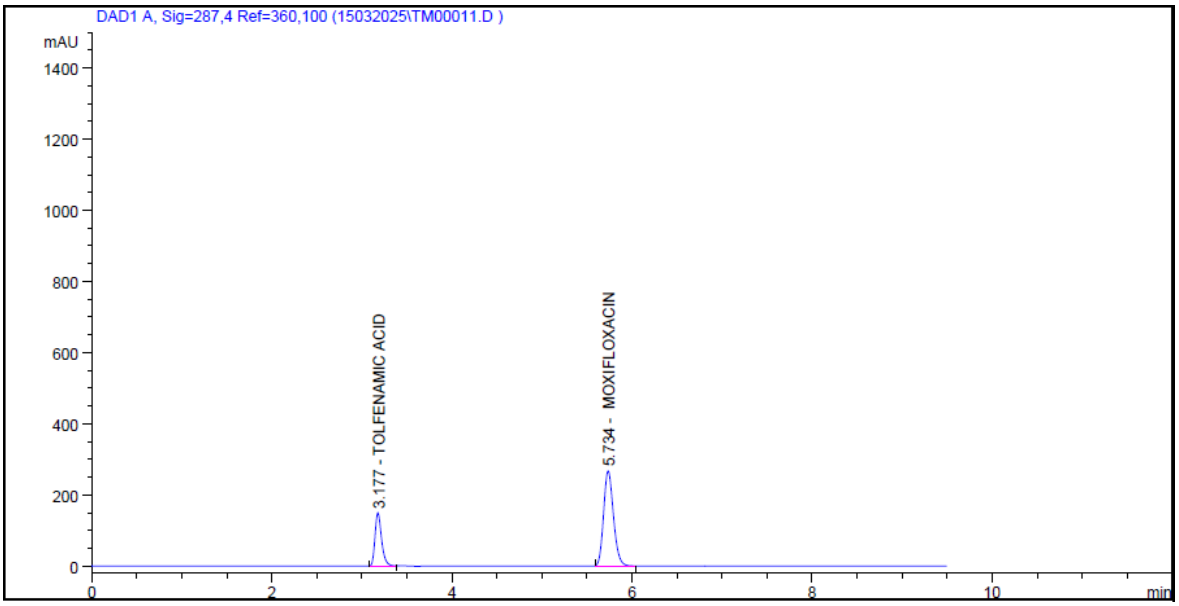
Fig.8 : Representative Chromatogram of Tolfenamic acid and Moxifloxacin using Methanol: 0.05% Acetic Acid (30: 70%v/v) 1 ml/ min.

Table.No.13. Details of chromatogram of Final Trials 05

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.762	172.60789	5650	0.70	-
2	5.237	612.73181	7356	0.69	6.63

- **The final chromatographic conditions selected were as follow:**

- ❖ Analytical column : Agilent C18 Column (250mm x 4.6mm, 5µm partical size).
- ❖ Injection volume : 20µl
- ❖ Flow rate : 1 ml/min
- ❖ Mobile phase : Methanol: water (0.05% Acetic acid) PH 4.2 with TEA (30 : 70v/v)
- ❖ Detection : 287 nm
- ❖ Run Time : 10 min



FigNo.9 : Chromatogram of standard Combination of Tolfenamic acid and Moxifloxacin

Table.No.18. Details of chromatogram of standard Combination containing Tolfenamic acid and Moxifloxacin

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.177	767.48016	9633	0.71	0.0000
2	5.734	2030.62598	13852	0.80	15.74

In the standard mixture of Tolfenamic acid and Moxifloxacin theoretical plates were found above 2000 i.e. for Tolfenamic acid and Moxifloxacin 9633 and 13852 at minimum RT 3.177 and 5.734 respectively**.**

5.1.5 Calibration experiment

- ❖ **RP-HPLC Method:**

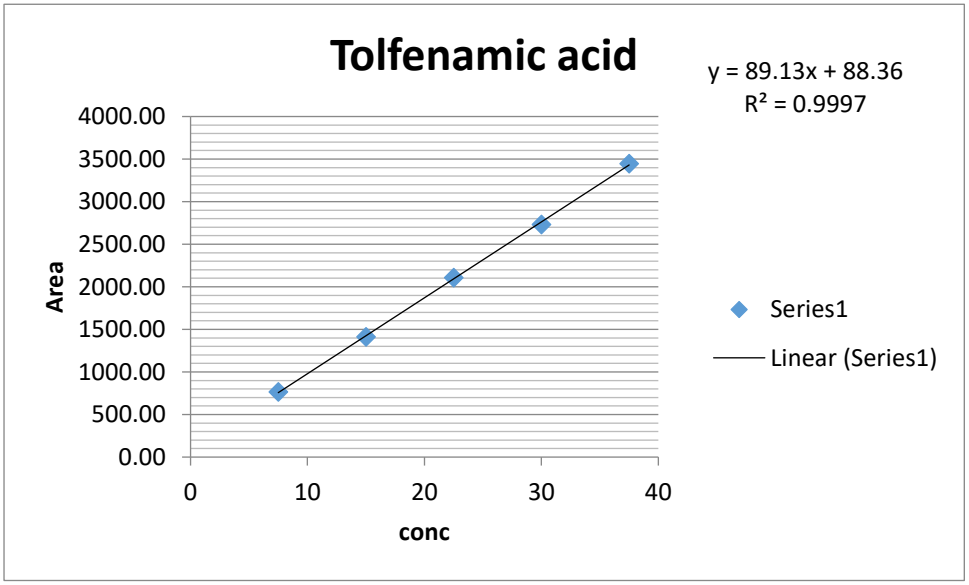
The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 7.5-37.5µg/mL for Tolfenamic acid and 15-75 µg/mL for Moxifloxacin (**Table No:22,and Table No:23)**depict the calibration data of Tolfenamic acid and Moxifloxacin .

The respective linear equation for Tolfenamic acid was y = 89.13x+88.36 and Moxifloxacin equation y = 111.3 x+373.7 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Tolfenamic acid and Moxifloxacin is depicted in (**Fig No.12 and Fig No.13).**

Table No 15: Linearity data for Tolfenamic acid

Method	**Conc µg/ml**	**Peak area(µV.sec)**		**Average peak area (µV.sec)**	**S.D. of Peak Area**	**% RSD of Peak Area**
		1	**2**			
RP-HPLC Method	7.5	767.48	766.58	767.03	0.64	0.08
	15	1407.29	1416.52	1411.91	6.53	0.46
	22.5	2107.23	2107.33	2107.28	0.07	0.00
	30	2724.57	2744.93	2734.75	14.40	0.53
	37.5	3448.73	3448	3448.37	0.52	0.01
	Equation		y = 89.13x + 88.36			
	R^2		0.999			

Fig.No.10: Calibration curve of Tolfenamic acid



The RP-HPLC Method for respective linear equation for Tolfenamic acid was y = 89.13 x= 88.36 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Tolfenamic acid is depicted in **Fig.16**

Table No 16: Linearity data for Moxifloxacin

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
RP-HPLC Method	15	2030.62	2033.90	2032.26	2.32	0.11
	30	3680.06	3678.48	3679.27	1.12	0.03
	45	5473.14	5466.03	5469.59	5.03	0.09
	60	7020.19	7058.49	7039.34	27.08	0.38
	75	8672.69	8729.08	8700.89	39.87	0.46
	Equation		y = 835.0+ 373.7			
	R^2		0.999			

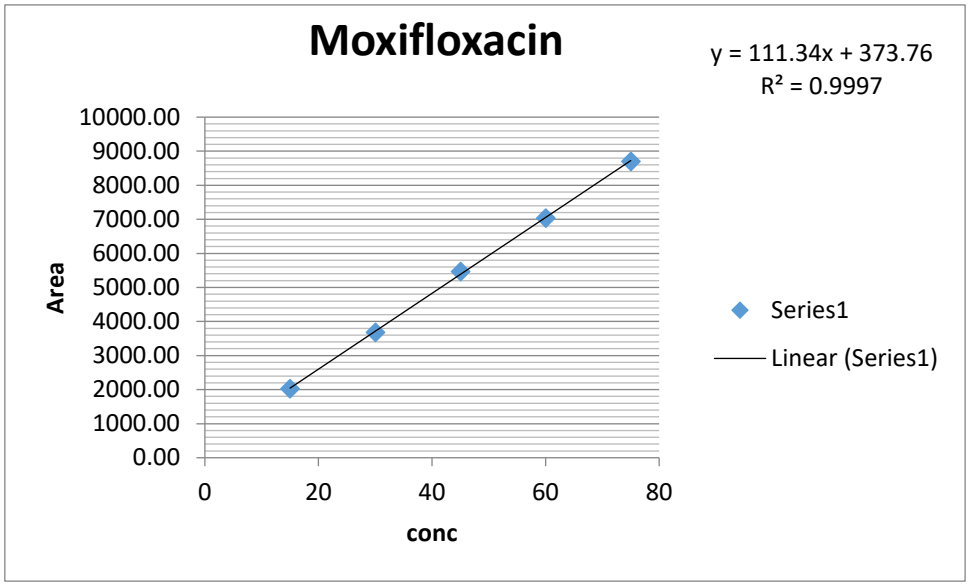


Fig.No.11: Calibration curve Of Moxifloxacin

The RP-HPLC method for respective linear equation for Moxifloxacin was y = 111.3 X+373.7 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Moxifloxacin is depicted in **Fig15.**

5.2. Analytical of Method Validation:

1. Linearity:

From Tolfenamic acid standard stock solution, different working standard solution (7.5-37.5µg/ml) were prepared in mobile phase Likewise from Moxifloxacin standard stock solution different working standard solution (15-75µg/ml) were prepared in mobile phase 20 µl of sample solution was injected into the chromatographic system using fixed volume loop injector. Chromatograms were recorded.

Table No 17. Linearity of Tolfenamic acid

Concentration µg/ml	Area Tolfenamic acid
7.5	767.03
15	1411.91
22.5	2107.28
30	2734.75
37.5	3448.00

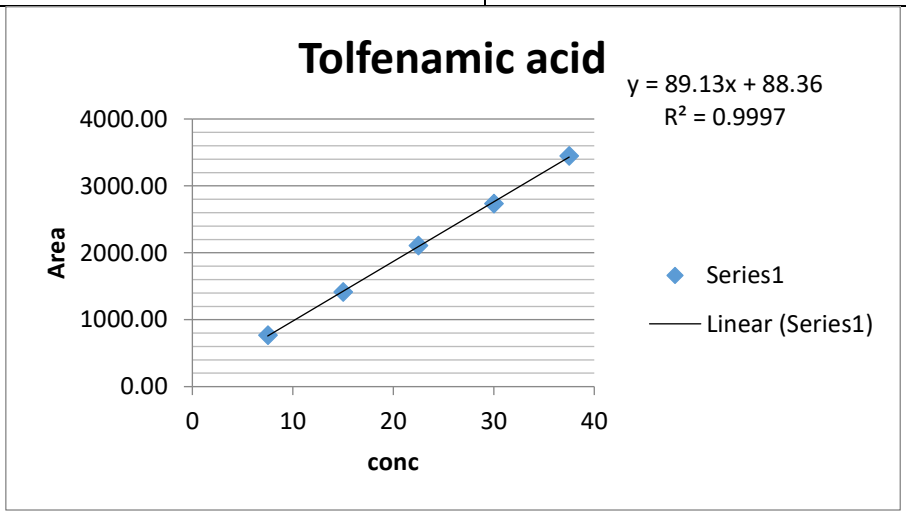


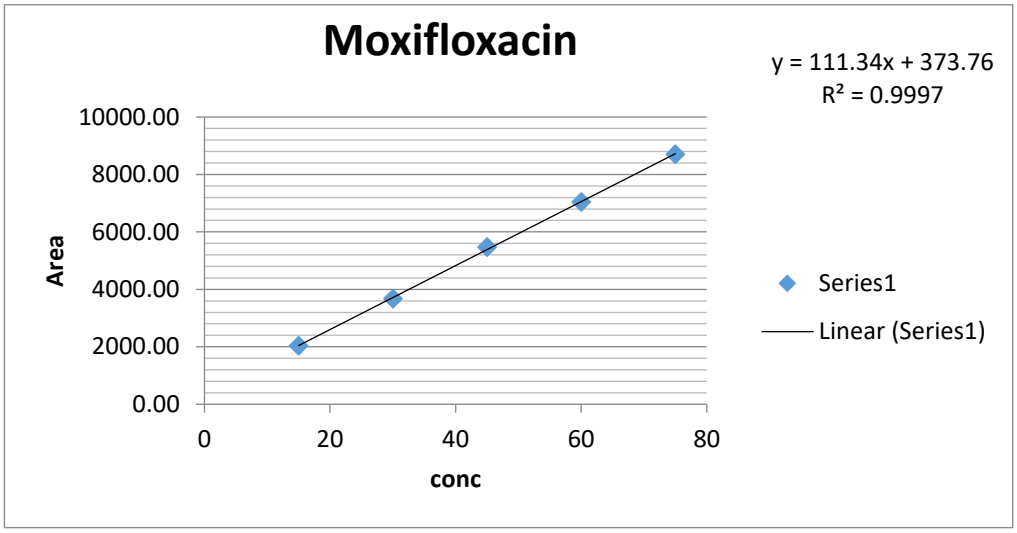
Fig.No.12. Calibration curve of Tolfenamic acid for HPLC method

Regression Equation Data Y=mx+c	
Slope(m)	89.13
Intercept(c)	88.36
Correlation Coefficient	0.999

Table No 18. Regression equation data for Tolfenamic acid

Table No 19. Linearity of Moxifloxacin

Concentration µg/ml	Area Moxifloxacin
15	2030.62
30	3679.27
45	5469.59
60	7039.34
75	8700.89



FigNo.13. Calibration graph of Moxifloxacin for HPLC method

Table.20. Regression equation data for Moxifloxacin

Regression Equation Data Y=mx+c	
Slope(m)	111.3
Intercept(c)	373.7
Correlation Coefficient	0.999

Linearity of of Tolfenamic acid and Moxifloxacin was observed in the range of 7.5-37.5 µg/ml and 15-75 µg/ml. Detection wavelength used was 287 nm.
The plot should be linear passing through the origin; Correlation Coefficient should not be less than 0. 999.that concluded

2. Accuracy: -

Recovery studies were performed to validate the accuracy of developed method. To pre analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed **.**

Table .21. Result of Recovery data for Tolfenamic acid and Moxifloxacin

METHOD	Drug	Level (%)	Amt. taken (µg/ml	Amt. Added (µg/ml	Area Mean* ± S.D.	Amt. recovered Mean *±S.D.	%Recovery Mean *± S.D.
RP-HPLC Method	TFM	80%	7.5	6	13.32±0.033	5.82±0.033	98.23±0.32
		100%	7.5	7.5	14.99±0.004	7.49±0.004	99.93±0.05
		120%	7.5	9	16.55±0.003	9.05±0.003	100.51±0.03
	MXF	80%	15	12	27.00±0.03	12.0±0.030	100.00±0.25
		100%	15	15	29.98±0.034	14.98±0.034	99.88±0.22
		120%	15	18	33.3±0.008	18.32±0.008	101.75±0.04

***mean of each 2 reading for RP-HPLC method**

Table.22. Statistical Validation of Recovery Studies Tolfenamic acid and Moxifloxacin

METHOD	Level of Recovery (%)	Drug	Mean % Recovery	Standard Deviation*	% RSD
Rp-HPLC Method	80%	TFM	98.23	0.32	0.32
		MXF	100.00	0.25	0.25
	100%	TFM	99.93	0.05	0.05
		MXF	99.88	0.22	0.22
	120%	TFM	100.51	0.03	0.03
		MXF	101.75	0.04	0.04

***Denotes average of three determinations for RP-HPLC** Accuracy of RP-HPLC method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 98-102%

3. System suitability parameters :(Repeatability)

To ascertain the resolution and reproducibility of the proposed chromatographic system for estimation of Tolfenamic acid and Moxifloxacin system suitability parameters were studied.

Table No.23: Repeatability studies on RP-HPLC for Tolfenamic acid and Moxifloxacin

Method	Concentration of Tolfenamic acid and Moxifloxacin (mg/ml)	Peak area	Amount found (mg)	% Amount found

RP-HPLC Method for TFM	22.5	2123.3	22.84	101.47
	22.5	2122.99	22.82	101.48
		Mean	**22.83**	**101.46**
		SD	**0.22**	**0.22**
		%RSD	**0.01**	**0.01**
RP-HPLC Method for MXF	45	5474.95	45.89	101.90
	45	5460.7	45.80	101.98
		Mean	**45.85**	**101.95**
		SD	**10.08**	**10.08**
		%RSD	**0.18**	**0.18**

Repeatability studies on RP-HPLC for Tolfenamic acid and Moxifloxacin was found to be 101.46 and 101.98%,The %RSD was less than 2%, which shows high percentage amount found in between 98% to 102% indicates the analytical method that concluded .

4. Precision: -

The method was established by analyzing various replicates standards of Tolfenamic acid and Moxifloxacin. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result that concluded.

Chromatogram of Precision:

Table No 24: Result of Intra day and Inter day Precision studies on RP-HPLC for Tolfenamic acid and Moxifloxacin

METHOD	**Drug**	**Conc^n (µg/ml)**	**Intraday Precision**		**Interday Precision**	
			Mean± SD	**%Amt Found**	**Mean± SD**	**%Amt Found**
Rp-HPLC METHOD	**MXF**	15	2054.04±5.38	100.92	2065.30±5.33	101.60
		45	5467.48±10.68	101.98	5479.87±0.52	102.23
		75	8722.32±24.04	100.28	8681.56±9.19	99.79
		7.5	763.16±3.54	100.95	762.21±0.11	100.82

	TFM	22.5	2125.54±2.83	101.58	2125.32±5.66	101.57
		37.5	3463.06±4.19	100.97	3430.95±3.14	100.01

***Mean of each 3 reading for RP-HPLC**

Intraday and Inter day Precision studies on RP-HPLC for Tolfenamic acid and Moxifloxacin which shows the high precision % amount in between 98% to 102% indicates to analytical method that concluded.

5. Robustness:

The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate, wavelength on retention time and tailing factor of drug peak was studied.

The mobile phase composition was changed in (±1 ml/min^{-1}**)** proportion and the flow rate was varied by (±1ml/min^{-1}**)**, and wavelength change (±1 ml/min^{-1}**)** of optimized chromatographic condition. The results of robustness studies are shown in **(Table No.66, 67**).Robustness parameters were also found satisfactory; hence the analytical method would be concluded.

Table No.25. Result of Robustness Study of Moxifloxacin

Parameters	**Conc.(µg/ml)**	**Amount of detected(mean ±SD**)	**%RSD**
Chromatogram of flow change 0.9ml	45	4479.04±27.00	0.60
Chromatogram of flow change 1.1 ml	45	3230.67±10.25	0.32
Chromatogram of comp change 29 ml Meoh+71ml Acetic acid Water	45	3814.8±11.35	0.30
Chromatogram of comp change 31 ml Methanol+69 ml Acetic acid Water	45	3847.39±26.76	0.70
Chromatogram of comp change wavelength change 249 nm	45	4235.7±2.41	0.06
Chromatogram of comp change wavelength change 251 nm	45	3464.52±27.29	0.81

Robustness Study of Moxifloxacin

The changes were did flow rate (±1 ml/ min^{-1}), mobile phase composition (±1 ml/ min^{-1}),and Wavelength (±1 ml/ min^{-1}) .%RSD for peak area was calculated which should be less than 2%.

Table No.26. Result of Robustness Study of Tolfenamic acid

Parameters	**Conc.(µg/ml)**	**Amount of detected(mean ±SD)**	**%RSD**
Chromatogram of flow change 0.9ml	15	1712.83±20.4	1.19
Chromatogram of flow change 1.1 ml	15	1210.32±6.92	0.57
Chromatogram of comp change 29 ml Meoh+71ml Acetic acid Water	15	1472.8±16.64	1.13
Chromatogram of comp change 31 ml Methanol+69 ml Acetic acid Water	15	1436.06±3.45	0.24
Chromatogram of comp change wavelength change 249 nm	15	1509.0±7.84	0.52
Chromatogram of comp change wavelength change 251 nm	15	1424.44±2.02	0.14

Robustness Study of Tolfenamic acid:

The changes were did flow rate (±1 ml/ min^{-1}), mobile phase composition (±1 ml/ min^{-1}),and Wavelength (±1 ml/ min^{-1}) .%RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded**.(Table No.56).**

6. Limit Detection

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope The limit of detection (LOD) may be expressed as:

$$\mathbf{LOD = 3.3\ (SD)/S}$$

where, SD = Standard deviation of Y intercept
S = Slope

Limit of detection = 0.1639 (µg/mL) of Tolfenamic acid
Limit of detection = 0.4484 (µg/mL)of Moxifloxacin

The LOD of Tolfenamic acid and Moxifloxacin was found to be 0.1639 (µg/mL) and 0.4484 (µg/mL), analytical method that concluded.

7. Limit Quantification

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope,
The quantitation limit (LOQ) may be expressed as:

LOQ = 10 (SD)/ S

where, SD = Standard deviation Y intercept
S = Slope
Limit of Quantitation = 0.49695 (µg/mL)
Limit of Quantitation = 1.3589 (µg/mL)

The LOQ of Tolfenamic acid and Moxifloxacin was found to be 0.4969 µg/mL) and 1.3589 (µg/mL), analytical method that concluded.

5. Analysis of tablet formulation:-
Procedure:

Weigh Bolus Tolfenamic acid and Moxifloxacin combination and calculated the accuracy weigh and transfer the sample equivalent to 25 mg. Tolfenamic acid and Moxifloxacin into 10 ml volumetric flask. Add about 10 ml Methanol of diluent and sonicate to dissolve it and make volume up to the mark with diluent. Mix well and filter through 0.45 µm filter. Further pipette 0.5 ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents. (37.5 µg/ml and 10 µg/ml). The simple chromatogram of test Tolfenamic acid and Moxifloxacin Shown in **(Fig No:54)** the amounts of Tolfenamic acid and Moxifloxacin were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated five times with Bolus formulation. Tablet Assay for %Lable claim for %RSD Calculated, Result was shown in **(Table No. 64).**

Brand Name: Duromoxi Bolus (Osvel pharma)
1 Bolus. = 2.7 gms
Eq. Wt for 15 mg = 15 x 2500/ 1500 = 25 mg
Take 25 mgs in 10 ml Methanol.= 750 µgm/ml TFM and 1500 µgm/ml MXF–II

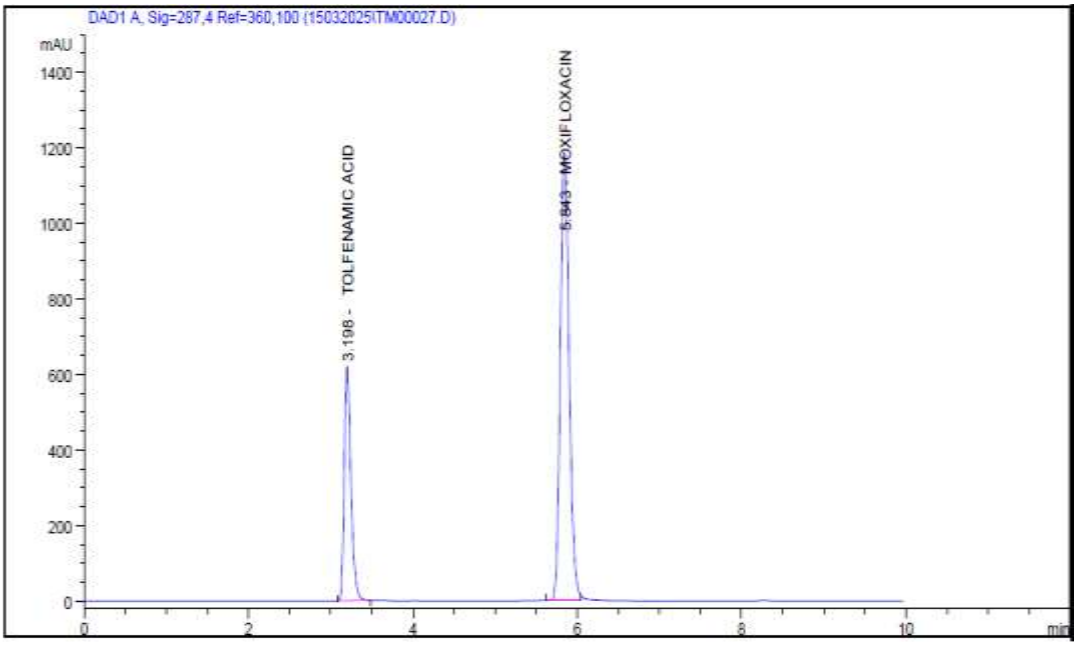


Fig No.14: Chromatogram for Marketed Formulation (37.5+75mcg)

Table.No.27. Details of Chromatogram of Marketed Formulation (37.5+75 mcg)

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.198	3477.43384	8161	0.69	0.0000
2	5.843	8833.85449	14725	0.81	15.80

Table.28. Analysis of marketed formulation.

Assay	Drug	Conc	Area	%Lable Claim	SD	%RSD
Rp-HPLC Method	**TFM**	37.50	3477.43	101.40	0.158	0.417
	MXF	75	8833.85	101.32	0.014	0.141
	TFM	37.50	3457.51	100.80	0.42	0.417
	MXF	75	8816.9	101.12	0.143	0.14

Analysis of marketed formulation were also %Lable Claim was found to be 98-101% Satisfactory are concluded. (**Table No. 63**).

9. Ruggedness

The degree of reproducibility of test result obtains by the analysis of same sample under variety of Condition. Such as different analyst, laboratory Different instrument**.**

Table.no. 29. Analysis of Analyst (30+60 mcg)

	R.T	AREA	TH.PLATES	SYMM
Analysis of Analyst-1 (30+60 mcg)	3.182	2726.85632	8325	0.65
	5.740	7022.45630	15263	0.81
Analysis of Analyst-II	3.190	2729.45200	8362	0.66
(30+60 mcg)	5.752	7024.78631	15723	0.82

5.3 Specificity and Selectivity

The analyses should have no interference from the extraneous components and be well resolved from them. Specificity is the procedure to detect quantitatively the analyst in presence of component that may be expected to be present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyst in presence of components that may be expected to be present in the sample matrix.

Table. No 30: Details of Chromatogram of Specificity and Selectivity

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.190	2120.57780	9563	0.72	-
2	5.702	5470.12304	15321	0.80	15.64

Conclusions for HPLC Method:

The developed method was validated according to the ICH guidelines. The linearity, precision, range, robustness was within the limits as specified by the ICH guidelines. Hence the method was found to be simple, accurate, precise, economic and reproducible. The proposed methods can be used for the routine quality control analysis Tolfenamic acid and Moxifloxacin in bulk drug as well as in formulationsThe method provides selective quantification of Tolfenamic acid and Moxifloxacin. This developed RP-HPLC method for estimation of Tolfenamic acid and Moxifloxacin is accurate, precise, robust and specific. The method has been found to be better than previously reported method, because of its less retention time, isocratic mode and use of an economical and readily available mobile phase, readily available column, UV detection and better resolution of peaks.

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