METHOD DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN AND AZITHROMYCIN IN PHARMACEUTICAL TABLET DOSAGE FORM

Dissertation work submitted to
The TamilNadu Dr. M.G.R. Medical University, Chennai
In partial fulfillment for the award of degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

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This is to certify that the dissertation work entitled **"METHOD DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LEVOFLOXACIN AND AZITHROMYCIN IN PHARMACEUTICAL TABLET DOSAGE FORM"** is a bonafide work of **ASHOK KUMAR BATHINI** carried out in RAINBOW PHARMA TRAINING LAB,HYDERABAD under my guidance andunder the supervision of T.SRINIVASA REDDY and has completed to my fullest satisfaction for partial fulfillment of the award of degree of **Master of Pharmacyin Pharmaceutical Analysis**, R.V.S college of Pharmaceutical Sciences,Sulur,Coimbatore,which is affiliated to The Tamilnadu Dr.M.G.R Medical University, Chennai. It is to certify that the part or whole of the work has not been submitted either to this university or any other university. This work is original and confidential.

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INTERNAL EXAMINER EXTERNAL EXAMINER

Place: Place: Date:

Date:

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ABBREVIATIONS

%	: Percentage
nm	: Nanometer
v/v	: Volume by volume
Min	: Minute
mg	: Milligram
µg	: Microgram
ng	: Nano gram
LC	: Liquid chromatography
HPLC	: High-performance liquid chromatography
GC	: Gas chromatography
UV	: Ultraviolet
HPTLC	: High-performance thin layer chromatography
TLC	: Thin layer chromatography
LC-MS	: Liquid chromatography-Mass spectroscopy
mm	: Millimeter
RSD	: Relative standard deviation
µ mol	: Micromole
RP-HPLC	: Reversed-phase high-performance liquid chromatography
RI	: Refractive index
USP	: United States Pharmacopoeia
HETP	: High equivalent theoretical plate

LOD	: Limit of detection
LOQ	: Limit of quantification
CV	: Coefficient of variance
S/N	: Signal to Noise
PDA	: Photo diode array detector
USFDA	: United state Food and Drug Administration
ODS	: Octa docile silane c_{18}
PPM	: Parts per million
WHO	: World Health Organization

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1. INTRODUCTION[1-8]

Introduction to Analytical Chemistry

Analytical Chemistry is defined as the "science and the art of determining the composition of materials in terms of the elements or compounds contained". This branch of chemistry, which is both theoretical, and a practical science, is practiced in a large number of laboratories in many diverse ways while analytical method, is a specific application of a technique to solve an analytical problem. Methods of analysis are routinely developed, improved and validated.collaboratively studied and applied. In analytical chemistry it is of prime importance to gain information about the qualitative and quantitative composition of substances and chemical species, that is, to find out what a substance is composed and exactly how much. In quantitative analysis the question is how much is present? The research work in this thesis is based on this criterion.

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. Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and environment. This instrumentation provides lower detection limits required to assure safe foods, drugs, water and air. Instrumental methods are widely used by Analytical chemists to save time, to avoid chemical separation and to obtain increased accuracy.

Most instrumental techniques fit into one of the four-principle areas mentioned below.

Spectrophotometric techniques

- UV and Visible Spectrophotometry
- Fluorescence and Phosphorescence Spectrophotometry
- Atomic Spectrophotometry (emission &absorption)
- Infrared Spectrophotometry

- Raman Spectrophotometry
- X-Ray Spectrophotometry
- Nuclear Magnetic Resonance Spectroscopy
- Mass Spectroscopy
- Electron Spin Resonance Spectroscopy

ElectrochemicalTechniques

- Potentiometry
- Voltametry
- Electrogravimetry
- Conductometry
- Amperomertry

Chromatographic Techniques

- High Performance Liquid Chromatography
- Gas chromatography
- High Performance Thin Layer Chromatography
- Thin Layer Chromatography
- GC- MS (Gas chromatography - Mass Spectroscopy
- LC-MS (Liquid Chromatography - Mass Spectroscopy)

Introduction to HPLC

High Performance Liquid Chromatography (HPLC) is one mode of chromatography, one of the most used analytical techniques. Chromatographic process can be defined as separationtechnique involving mass-transfer between stationary and mobile phase. HPLC utilises a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent,

and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components.

The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

Principle

Liquid chromatography is a separation method in which the components of a sample partition between two phases – one of these phases is a stationary bed with a large surface area and the other is a liquid which percolates through the stationary bed. The sample is carried by the mobile liquid phase through the column. Samples partition (equilibrate) into the stationary liquid phase, based on their solubility in the phases and/ or molecular size solubilities. The compounds of the sample separate from one another based on their affinities for the stationary bed. This type of chromatography process is called elution.

Theory

HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porouspacking beads, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process:

1)Hydrophobic (non-specific) interactions are the main ones in reversed-phase (RP) separations.

2) Dipole-dipole (polar) interactions are dominant in normal phase (NP)**.**

3)Ionic interactions are responsible for the retention in ion-exchangechromatography.

All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface. The weaker the eluent interaction, the longer the analyte will be retained on the surface. SEC (size-exclusion chromatography)is another case. It is the separation of the mixture by the molecular size of its components. The basic principle of SEC separation is that the bigger the molecule, the less possibility there is for it to penetrate into the adsorbent pore space. So, the bigger the molecule

Stationary Phases (Adsorbents)

HPLC separations are based on the surface interactions, and depend on the types of theadsorption sites. Modern HPLC adsorbents are the small rigid porous particles with high surface area.

Main adsorbent parameters are:

Particle size: 3 to 10 μm

Particle size distribution: as narrow as possible, usually within 10% of the mean;

The last parameter in the list represents an adsorbent surface chemistry.

Depending on the type of the ligand attached to the surface, the adsorbent could be normal phase (-OH, -NH2), or reversed-phase (C5, C8, C 18 CN, NH2), and even anion (CH2NR3+OH-), or cation (R-SO3\-H+) exchangers.

Mobile Phases

In HPLC, the type and composition of the eluent is one of the variables influencing the separation. Despite the large variety of solvents used in HPLC, there are several common properties:

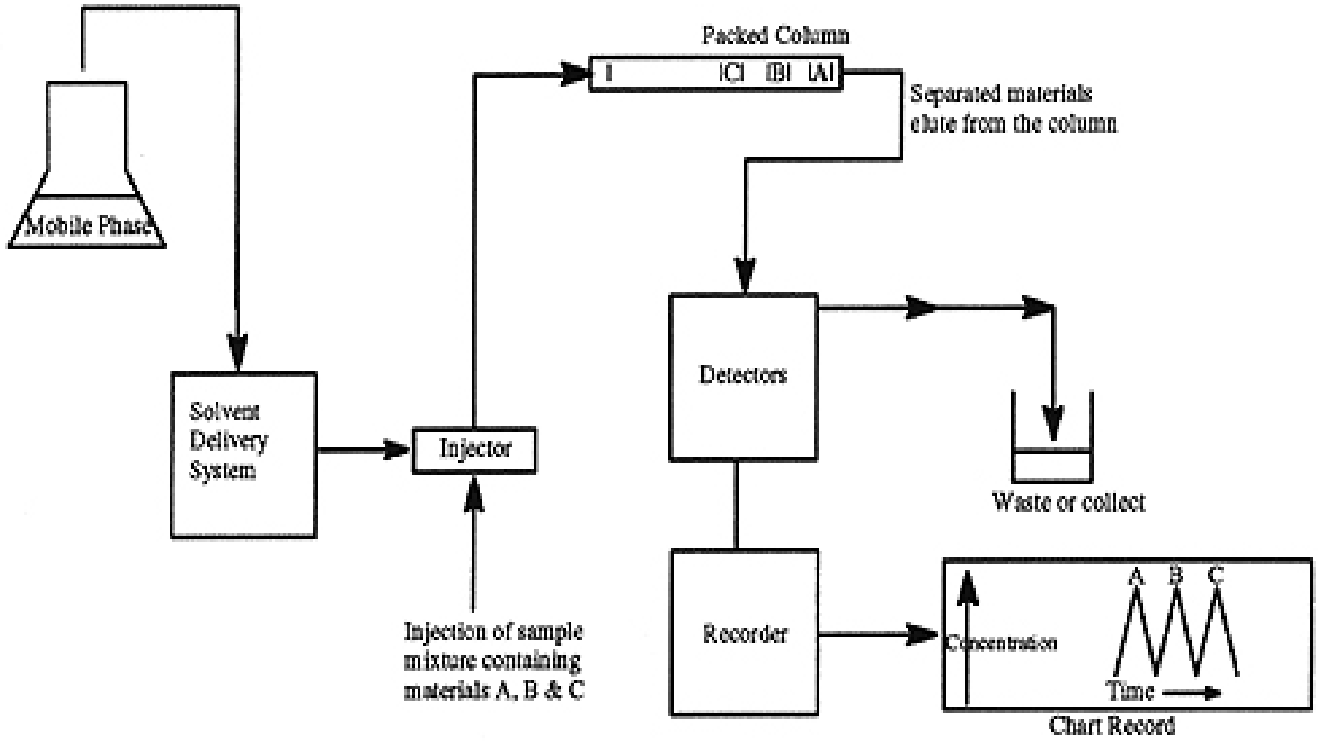
- Purity
- Detector compatibility
- Solubility of the sample

- Low viscosity
- Chemical inertness

For normal phase mode, solvents are mainly nonpolar; for reversed-phase, eluents are usually a mixture of water with some polar organic solvent such as acetonitrile or methanol. Size-exclusion HPLC has special requirements. SEC eluents have to dissolve polymers, but the most important is that SEC eluent has to suppress possible interactions of the sample molecule with the surface of the packing material.

Instrumentation of HPLC system

Block diagram showing the components of an HPLC instrument



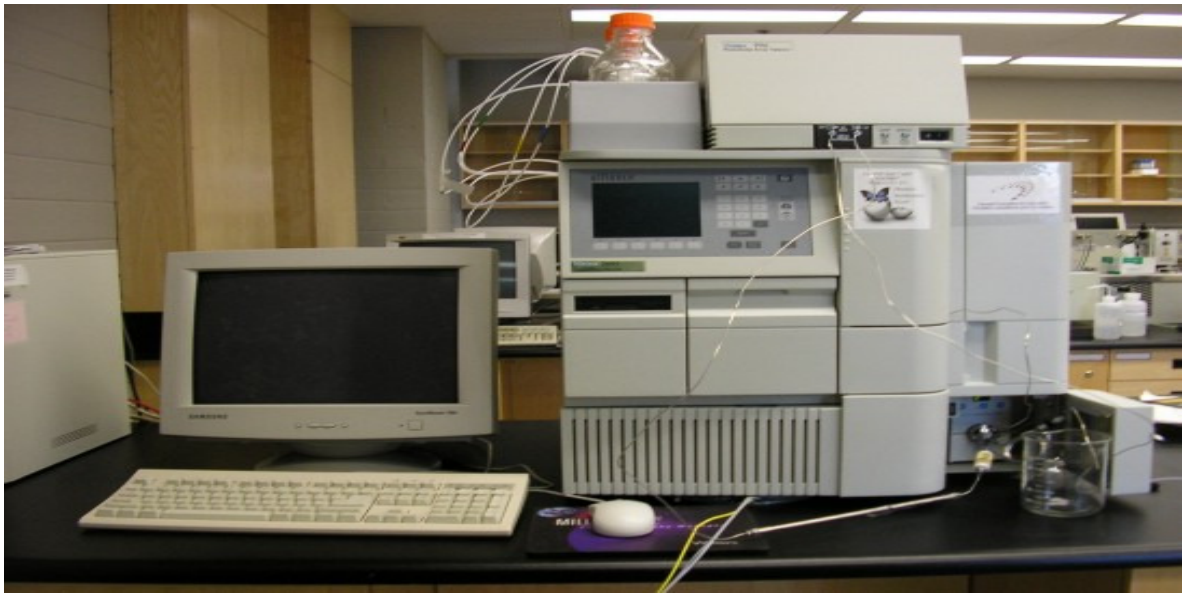


Fig 2: Pictorial Representation of HPLC Instrument

HPLC instrumentation includes a pump, injector, column, detector and data system. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometre size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on the recorder.

Detection of the eluting components is important, and this can be either selective or universal,depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyse the chromatographic data, computer, integrator, and other data processing equipment arefrequently used.

Components of HPLC:

Mobile phase reservoir, filtering

Pump

Injector

Column

Detector

Data system

Mobile Phase Reservoir, Filtering

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium is not sufficient for degassing aqueous solvents. It is useful to apply a vacuum for 5-10 min. and then keep the solvent under a helium atmosphere.

Pump

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.

The most important advantages are: higher resolution, faster analyses, and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle packings that require less pressure. Flow rate stability is another important pump feature that distinguishes pumps. Very stable flow rates are usually not essential for analytical chromatography. However, if the user plans to use a system in size exclusion mode, then there must be a pump which provides an extremely stable flow rate.

An additional feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively,

this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps include such stateof- the-art technology as electronic feedback and multiheaded configurations.

Modern pumps have the following parameters:

- Flow rate range: 0.01 to 5 mL/min
- Flow rate stability: not more than 1%
- For SEC flow rate stability should be less than 0.2%
- Maximum pressure: up to 300 hPa.
- It is desirable to have an integrated degassing system, either helium purging,
- or membrane filtering.

Injector

Sample introduction can be accomplished in various ways. The simplest method is to use aninjection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of autosamplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, loss in efficiency or all of these. It is always best to remove particles from the sample by filtering over a 5 μm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns.

Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance. Typical sample mass with 4.6 mm ID columns range from the nanogram level up to about 2 mg

diluted in 20 ml of solvent. In general, it will be noted that much less sample preparation is required in LC than in GC since unwanted or interfering compounds, or both, may often be extracted, or eliminated, by selective detection.

Column

The HPLC Column holds the stationary phase for separating the components of the sample. The columns are usually made up of SS-316 grade steel. Apart from columns, the material of construction of tubing and fittings, plumbing and connections are also very critical. Apart from resistivity to corrosion, connections and plumbing should have a very low dead volume. Many different reverse phase columns will provide excellent specificity for any particular separation.

It is therefore best to routinely attempt separations with a standard C_8 or C_{18} column and determine if it provides good separations. If this column does not provide good separation or the mobile phase is unsatisfactory, alternate methods or columns should be explored. Reverse phase columns differ by the carbon chain length,degree of end capping and percent carbon loading.Diol, cyano and amino groups can also be used for reverse phase chromatography.

Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 μm) particles. The internal diameter of the columns is usually 4.6 mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed. Packing the column tubing with small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment.

In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when

new, and to retain the chromatogram. If questionable results are obtained later, the test mixture can be injected again under specified conditions. The two chromatograms may be compared to establish whether or not the column is still useful.

Detector

Today, optical detectors are used most frequently in liquid chromatographic systems. These detectors pass a beam of light through the flowing column effluent as it passes through a lowvolume (~ 10 µl) flow cell. The variations in light intensity caused by UV absorption, fluorescence emission or change in refractive index, from the sample components passing through the cell, are monitored as changes in the output voltage. These voltage changes are recorded on a strip chart recorder and frequently are fed into a computer to provide retention time and peak area data.

The following are the different detectors used in HPLC.

Refractive Index (RI) detectors

measure the ability of sample molecules to bend or refract light. This property for each molecule or compound is called its refractive index. For most RI detectors, light proceeds through a bi-modular flow-cell to a photodetector. One channel of the flow-cell directs the mobile phase passing through the column while the other directs only the mobile phase. Detection occurs when the light is bent due to samples eluting from the column, and this is read as a disparity between the two channels.

Ultra-Violet (UV) Detectors

measures the ability of a sample to absorb light. This can be accomplished at one or several wavelengths: A Fixed Wavelength measures at one wavelength, usually 254 nm. Variable Wavelength measures at one wavelength at a time, but can detect over a wide range of wavelengths. Diode Array measures a spectrum of wavelengths simultaneously.UV detectors have a sensitivity to approximately 10-8 or 10 -9 gm/ml.

Fluorescent Detectors

measure the ability of a compound to absorb then re-emit light at given wavelengths. Each compound has a characteristic fluorescence. The excitation source passes through the flow-cell to a photodetector while a monochromator measures the emission wavelengths. Has sensitivity limit of 10-9 to 10-11 gm per mL.

RadiochemicalDetection

involves the use of radiolabeled material, usually tritium (3H) or carbon-14 (14C). It operates by detection of fluorescence associated with beta-particle ionization, and it is most popular in metabolite research.

Data System

Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time. The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention. There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent processors in chromatographs are found in several areas. First, additional automation options become easier to implement. Secondly, complex data analysis becomes more feasible. These analysis options include such features as run parameter optimisation and deconvolution (i.e. resolution) of overlapping peaks. Finally, software safeguards can be designed to reduce accidental misuse of the system.

SELECTION OF CHROMATOGRAPHIC MODE

Reversed-phase chromatography (RPC), the most common mode for small organic molecules. Note that ionizable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analytes in a non-ionized state) or with ion-pairing reagents. In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix.

CHOICE OF THE COLUMN

Selection of the column is the first and the most important step in method development. The appropriate choice of separation column indicates three different approaches.

- Selection of separation.
- The particle size and nature of the column packing.
- The physical parameters of the column i.e. the length and the diameter.

Some of the important parameters considered while selecting chromatographic columns are

- Length and diameter of the column
- Packing material
- Shape of the particles
- Size of the particles
- Pore volume
- Surface area

Reversed phase mode of chromatography facilities a wide range of columns covering wide range of polarities by cross linking silanol groups with alkyl chains like like dimethyl

silane (C_2), butylsilane (C_4), octylsilane(C_8), octadecylsane (C_{18}),base deactivated silane (C_{18}), phenyl, cyanoproply (CN), nitro, amino etc.

Silica based columns with different cross linkings in the increasing order of Polarity are as follows:

<……. Non-polar …… moderately polar……. Polar. ……>

C_{18}< C_8< C_6< Phenyl < Amino < Cyano<nitrile < Silica

SELECTION OF MOBILE PHASE

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all impurities and degradants from each other and from analytepeak.In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute-stationary phase, solute-mobile phase, and mobile phase-stationary phase.

The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation). Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pka of the analytes. The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer and its strength
- pH of the buffer or pH of the mobile phase
- Selection of buffer
- Mobile phase composition

A. BUFFERS, IF ANY, AND IT'S STRENGTH

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most commonly employed buffers are

- Phosphate buffers prepared using salts like KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4etc
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers-Ammonium acetate, Sodium acetate etc.
- Acetic acid buffers prepared using CH_2COOH.

The retention also depends on the molar strengths of the buffer-molar strength is inversely proportional to the retention times. Ideally the strength of the buffer shall be opted between 0.01M to 0.20M. After selecting the strength of the buffer, it can be varied by about 10-20% and the effect of variation was studied and it should be rugged for at least 2% variation in strength. It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This is due to the fact that the silioxane linkages are cleaved below pH 2.0, while pH values above 8.0 the silica may dissolve.

B. pH OF THE BUFFER

pH plays an important role as it controls the elution properties by controlling the ionization characteristics. In RP-HPLC the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. So, acid shows decrease in retention with increasing pH while base show increase in retention.

C. SELECTION OF BUFFER

Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. Almost all of the pH related change in retention occurs for pH values within ±1.5 units of pKa value. Outside this range the compound is either ionized or unionized, and its retention doesn't change much with pH.

The relationship between RPC retention and mobile phase pH is more complicated for compounds that contain multiple acidic and/or basic groups. If these groups are all same (acidic, basic) retention as a function of pH is similar. Buffer strength of 10-50 mM are generally adequate, but 25mM are compromise and suitable. The buffers showing UV absorbance below 220 nm were preferable. An experiment was conducted using buffers having different pH to obtain the required separations. After reviewing the results, the pH was selected which is rugged for at least ±0.2 units of the selected pH.

D. MOBILE PHASE COMPOSITION

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to the fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and acetonitrile. Experiments should be conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations of analyte peak. A mobile phase which gives separation of analyte peak and which is rugged for variation of both aqueous and organic phase by at least ± 0.2% of the selected mobile phase composition was used.The compounds can be further classified as neutral or ionic. Samples classified as ionic include acids, bases, amphoteric compounds and organic salts. If the sample is neutral, buffers or additives are generally not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase.

SELECTION OF FLOW RATE

Generally flow rate shall not be more than 2.0 ml/min. the flow rate shall be selected based on the following data.

- Retention time

- Column back pressure
- Resolution between the peaks
- Peak symmetries

The flow rate which gives least retention times, good peak symmetries, least back pressures and better separation will be selected.

SELECTION OF DETECTOR

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, florescence, conductance, oxidation, reduction etc. The characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- ✓ High sensitivity facilitating trace analysis
- ✓ Negligible baseline noise to facilitate lower detection
- ✓ Large linear dynamic range
- ✓ Low dead volume
- ✓ Ease in calibration and standardization
- ✓ Inexpensive to purchase and operate

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful. Generally LC equipped with PDA detector was the first choice. UV source like mercury vapour lamp is most widely used because majority of the compounds of pharmaceutical interest are absorbs at 250 – 270 nm and this lamp has an intense line spectrum at this region.

OPTIMIZATION OF HPLC METHOD

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts, asymmetry, capacity factor, elution time, detection limits, limit of quantitation and overall ability to quantify the specific analyte of interest.

Optimization of a method can follow either of two general approaches:

- Manual
- Computer driven

The manual approach involves varying one experimental variable at a time, while holding all other constant and recording changes in response.

The variables might include flow rate, mobile or stationary phase composition, temperature, detection wavelength and P^H.

This univariate approach to system is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables.

In the second approach, computer driven automated method development, efficiency is optimized while experimental input is minimized. Computer driven automated approaches can be applied to many applications. In addition, they are capable of significantly reducing the time, energy and cost of all instrumental method development.

VALIDATION[9]

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications.

Validation is a basic requirement to ensure quality and reliability of the results for all analytical applications.

Validation is defined as follows by different agencies:

European Committee (EC)

Action of providing in accordance with the principles of good manufacturing practice that any procedure, process, equipment, material, activity or system actually leads to the expected results. In brief validationis a key process for effective Quality Assurance

Food and Drug Administration (FDA)

Provides a high degree of assurance that specific process will consistently produce a product meeting its predetermined specification and quality attributes.

World Health Organization (WHO)

Action of providing that any procedure, process, equipment, material, activity, or system actually leads to the expected results.

Objective of Validation

There are two important reasons for validating assays in the pharmaceutical industry. The first, and by for the most important, is that assay validation is an integral part of the quality-control system.

The second is that current good manufacturing practice regulation requires assay validation. In industry it would be difficult to confirm that the product being manufactured is uniform and that meet the standards set to assure fitness for use. The varying nature of the differences between the analytical development laboratory and quality control laboratory is a good reason for validation program.

Method Validation[10]

Method validation is the process of proving that an analytical method is acceptable for its intended purpose. For pharmaceutical methods, guidelines from the United States Pharmacopoeia (USP), International Conference on Harmonization (ICH), and the Food and Drug Administration (FDA) provide a framework for performing such validations. In general, methods for regulatory submission must include studies on specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, and robustness.

Analytical Method needs to be Validated or Revalidated

- Before their introduction into routine use. Whenever the condition changes for which the method has been validated. Example: sample with a different matrix, an instrument with different characteristics.
- Whenever the method is changed and changes are outside the original scope of the method.

Benefits of Method Validation[8]

A fully validated process may require less in-process control and end product testing. It deepens the understanding of processes, decrease the risks of processing problems, and thus assure the smooth running of the process**.**

VALIDATION PARAMETERS

Accuracy

Precision

Specificity

Limit of detection

Limit of quantification

Linearity and range

Robustness

System suitability

Accuracy

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the values, which is, accepted either as a conventional, true or accepted reference value, and the value found.

Precision

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree.

The measured standard deviation can be subdivided into 3 categories:

- Repeatability
- Intermediate precision
- Reproducibility

Repeatability

Repeatability is obtained when the analysis is carried out in a laboratory by an operator using a piece of equipment over a relatively short time span. At least 6 determinations of 3 different matrices at 2 or 3 different concentrations should be performed, and the RSD is calculated.

Intermediate Precision

Intermediate precision is a term that has been defined by ICH (4) as the long-term variability of the measurement process. It is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method's intermediate precision may reflect discrepancies in results obtained

- From different operators,
- From inconsistent working practice (thoroughness) of the same operator,
- From different instruments,
- With standards and reagents from different suppliers,

- With columns from different batches or
- A combination of these.

Specificity

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.

Reproducibility

Reproducibility is defined by the precision obtained between different results laboratories. The objective is to verify that the method will provide the same in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts, and by using operational and environmental conditions that may differ from, but are still within, the specified parameters of the method (inter laboratory tests). Validation of reproducibility is important if the method is to be used in different laboratories.

Robustness

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

Examples of typical variations are:

- Stability of analytical solutions
- Extraction time In the case of liquid chromatography, examples of typical variations

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate

Limit of Detection (LOD)

The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified.

A. Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

B. Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A s/n ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

C. Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

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

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

Limit of Quantification (LOQ)

The limit of quantification is defined as the lowest concentration of an analyte in sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

LINEARITY

Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure.

RANGE

Range is the interval between the upper and lower concentration of the analyte in the sample for which it has a suitable level of precision, accuracy and linearity.

SYSTEM SUITABILITY

According to the USP, system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, columns, analysts) is suitable for the intended application.

2. LITURATURE REVIEW

- **Srinivas N[11],et al** A highly selective, sensitive and accurate HPLC method has been developed and validated for the estimation of three fluoroquinolones (FQs) viz., gatifloxacin (GFC), sparfloxacin (SFC) and moxifloxacin (MFC) with 500 microL human plasma using levofloxacin (LFC) as an internal standard (IS). The sample preparation involved simple liquid-liquid extraction of GFC, SFC, MFC and IS from human plasma with ethyl acetate. The resolution of peaks was achieved with phosphate buffer (pH 2.5)-acetonitrile (80:20, v/v) at a flow rate of 1 mL/min on a KromasilC(18) column. The total chromatographic run time was 18.0 min and the simultaneous elution of GFC, SFC, MFC and IS occurred at approximately 10.8, 12.8, 17.0 and 6.0 min, respectively. The method proved to be accurate and precise at linearity range of 100-10,000 ng/mL with a correlation coefficient (r) of > or =0.999. The limit of quantitation for each of the FQs studied was 100 ng/mL. The intra- and inter-day precision and accuracy values found to be within the assay variability limits as per the FDA guidelines. The developed assay method was applied to a pharmacokinetic study in human volunteers following oral administration of 400 mg GFC tablet.

- **Satish A. Patel[12],et al** A simple and sensitive high performance thin layer chromatographic method has been developed and validated for the simultaneous estimation of the levofloxacin and ornidazole in combined dosage forms. The stationary phase used was precoated silica gel 60F 254 plate. The mobile phase used was a mixture of n-butanol: ethanol: ammonia (8 M) (5:0.5:1.5, v/v/v). The detection of spots was carried out densitometrically using a UV detector at 310 nm in absorbance mode. This system was found to give compact spots for levofloxacin (Rf value of 0.31 ± 0.003) and ornidazole (Rf value of 0.83 ± 0.008). The method was validated in terms of linearity, accuracy, precision, limit of detection, limit of quantification. The calibration curve was found to be linear between 40 to 140 and 80 to 280 ng/spot for levofloxacin and ornidazole, respectively with significantly high value of correlation coefficient (r 2 > 0.99). The limits of detection and quantitation were found to be 9.98 and 30.25 ng/spot, respectively for levofloxacin and 21.12 and 64.01 ng/spot, respectively for ornidazole.

- **M. Senthil Raja[13],et al** A simple reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of Azithromycin and Ambroxol Hydrochloride in combined dosage form. The separation was carried out using a mobile phase consisting of acetonitrile and mono basic potassium phosphate buffer of pH 8.5 in the ratio of 65:35 v/v. The column used was C18 phenomenex Gemini 5m, 250cm x 4.6mm id with flow rate of 2ml/min using PDA detection at 220nm. The described method was linear over a concentration range of 96-145mg/ml and 80-125mg/ml for the assay of Azithromycin and Ambroxol Hydrochloride respectively. The retention times of Ambroxol and Azithromycin were found to be 3.7min and 6.1min respectively. Results of analysis were validated statistically and by recovery studies. The limit of quantification (LOQ) for Azithromycin and Ambroxol Hydrochloride were found to be 96.7mg/ml and 8.35mg/ml respectively. Then the limit of detection (LOD) for Azithromycin and Ambroxol Hydrochloride were found to be 31.91 mg/ml and 2.75 mg/ml respectively.

- **M. Sudheer[14], et al** A sensitive and specific isocratic RP-HPLC was developed for quantitative estimation of Azithromycin and AmbroxolHCl tablet formulation. The developed method consisting the mobile phase K2HPO4 – pH 6.5 : (68 : 32) with isocratic programming, Hypersil, BDS, C 8, column (150 mm x 4.6 mm i.d., 5µm particle size) as stationary phase with a flow rate of 1.5 mL/minute by using λmax 215nm and PDA detector. Proposed method was found to be linear in the concentration range of 100.0 to 360.0 µg/mL for Azithromycin and 15.0 to 54.0µg/mL for AmbroxolHCl respectively, the correlation coefficient was found to be 0.999. Precision study showed that the percentage relative standard deviation was within the range of acceptable limits, and the mean recovery was found to be 100.36 % for Azithromycin and 100.24% for AmbroxolHCl. The developed method was validated for specificity by stress studies. AmbroxolHCl and Azithromycin were subjected to stress condition and productswere analyzed by using photo diode array detector. It was found to be stable in milder condition of stress (0.1 M HCl, 0.1 M NaOH, 3% H2O2, at 60°C/10 minutes). The analyte peaks were well resolved from the degraded impurities.

- **Mocho´n[15],et al** A spectrofluorimetric method to determine levofloxacin is proposed and applied to determine the substance in tablets and spiked human urine and serum. The fluorimetricmethod allow the determination of 20–3000 ng ml_1 of levofloxacin in aqueous solution containing acetic acid–sodium acetate buffer (pH 4) with lexc_292 and lem_494nm, respectively. Micelle enhanced fluorescence improves the sensibility and allows levofloxacin direct measurementin spiked Human serum (5 mg ml_1) and urine (420 mg ml_1), in 8 mM sodium dodecyl sulphate solutions at pH 5.

- **NájlaMohamad Kassab[16],et al** The objective of this research was to develop and validate an alternative analytical method for quantitative determination of levofloxacin in tablets and injection preparations. The calibration curves were linear over a concentration range from 3.0 to 8.0 μg mL^{-1}. The relative standard deviation was below 1.0% for both formulations and average recovery was 101.42 ± 0.45% and 100.34 ± 0.85% for tablets and injection formulations, respectively. The limit of detection and limit of quantitation were 0.08 and 0.25 μg mL^{-1}, respectively. It was concluded that the developed method is suitable for the quality control of levofloxacin in pharmaceuticals formulations.

- **BN Suhagia[17],et al** A simple and sensitive spectrophotometric method has been developed for determination of azithromycin in its pharmaceutical dosage forms. In the proposed method, azithromycin is oxidized with potassium permanganate to liberate formaldehyde, which is determined in situ using acetyl acetone, in the presence of ammonium acetate. A yellow colouredchromogen was obtained, having an absorption maxima at 412 nm. The method is found to be linear in the concentration range of 10-75 μg/ml, with regression coefficient of 0.9978. Various reaction parameters such as concentration of potassium permanganate and reagent, time required for oxidation, and maximum colour intensity were optimized. The method was validated, and can be used successfully to assay azithromycin in its pharmaceutical dosage forms viz. tablets, capsules, and injections.

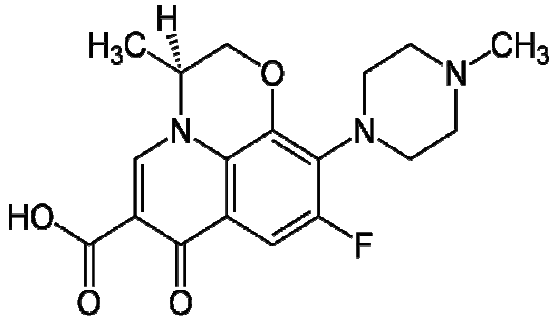
- **Sachin Kumar Singh[18],et al** A RP-HPLC method was developed and validated for quantitative determination of azithromycin in pharmaceutical suspension dosage forms. The chromatography was carried out on a Phenomenex C 18 (150 x 4.6 mm i.d., 5µ) column with Acetonitrile: 0.5 % Formic acid as mobile phase (Isocratic A: B = 40: 60 % v/v), at 215 nm detector wave length with a flow rate of 1 ml/min. Clarithromycin was used as an internal standard. The linearity was established in the range of 20 - 600 ng/ml for HPLC. The HPLC method was accurate and precised for azithromycin suspension with a recovery of 98.75 to 99.44%. The spiked sample solutions were stable upto 1 month. This validated method can be used for estimation of azithromycin in pharmaceutical suspension.

2.1DRUG PROFILE

LEVOFLOXACIN

Molecular formula :$C_{18}H_{20}FN_3O_4$

Structure :Levofloxacin



Chemical name :(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperzin -1-yl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid

Molecular weight : 361.3675

Solubility:soluble in water and methanol

Melting range : 218 ºC

Pka:5.5 to 7.5

Category :Anti-Bacterial Agents,

Quinolones,

Nucleic Acid Synthesis Inhibitors,

Anti-Infective Agents.

Pharmacokinetic data

Bioavailability **:**99%

Protein binding **:**24 to 38%

Metabolism **:**Renal

Half life:6 to 8 hours

Excretion **:**Urinary

Dosage form **:**tablet

Dose :250 mg to 750 mg

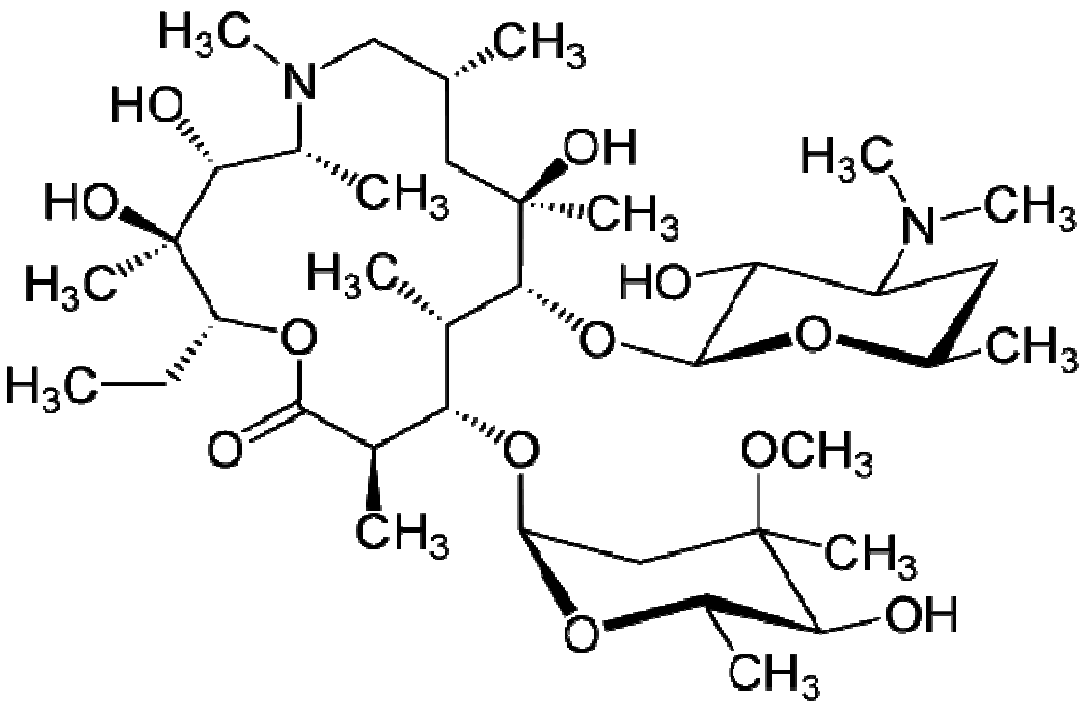
Mechanism of action **:**

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 is inhibited.

AZITHROMYCIN

Molecular formula : $C_{38}H_{72}N_2O_{12}$

Structure **:** Azithromycin



Chemical name : (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-15-oxo- 11-{[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-]oxy}-1-oxa-6-azacyclopentadec-13-yl 2,6-dideoxy-3-C-methyl-3-O-methyl-α-L-ribo-hexopyranoside

Molecular weight **:**748.9845

Solubility:rapidly soluble in methanol , Acetonitrile and partially Soluble in water

Melting range : 113–115◦C

Pka:8.74

Category : Anti-Bacterial Agents

Other Macrolides

Macrolides

Pharmacokinetic data

Bioavailability **:** 38% for 250 mg capsules

Protein binding **:** decreasing from 51% at 0.02 µg/mL to 7% at 2 µg/mL.

Metabolism **:** Hepatic

Half life **:** 68 h

Excretion **:** Biliary, Renal (4.5%)

Dosage form **:** tablet

Dose **:**adult dose is 500-2000 mg

Mechanism of action **:**

Azithromycin prevents bacteria from growing by interfering with their protein synthesis. It binds to the 50S subunit of the bacterial ribosome, and thus inhibits translation of mRNA. Nucleic acid synthesis is not affected.

3. AIM & OBJECTIVE

The literature review had showed that there are few individual reported methods for the determination of Levofloxacin and Azithromycin by RP-HPLC in pharmaceutical dosage forms.But there is no evidence available for simultaneous estimation for the above combination by RP-HPLC. Hence an attempt was made to develop a simple, precise, accurate, and economical RP-HPLC method for determination ofLevofloxacin and Azithromycin in pharmaceutical formulation.

PLAN OF WORK:

To develop a new RP-HPLC method for the estimation of LevofloxacinandAzithromycin pharmaceutical dosage form.

- Study of physico chemical properties of drug(pH, pka,solubility, etc.,)
- Selection of chromatographic condition (Mobile phase, column, flow rate, etc.,)
- Optimization of method.
- Study of system suitability parameters.
- Validation of proposal method.
- Applying developed ,method to marketed formulation

To validate the developed method by determining the validation parameters like Accuracy,

- Precision,
- Linearity,
- Limit of detection,
- Limit of quantification,
- Robustness,
- System suitability,
- Force degradation studies.

4. EXPERIMENTAL PART

4.1 MATERIALS AND INSTRUMENTS

Drug samples

Levofloxacin and Azithromycinwere generously given by Rainbow pharma training laboratories, Hyderabad.

Table no. 4.1 purity of working standards:

Drug name	Purity
Levofloxacin	99%
Azithromycin	99%

Tablets used :

BRAND: LEVOMAC-AZ

Each tablet contains:

Levofloxacin - 500mg and

Azithromycin - 500mg

Chemicals and solvents used :

Water for HPLC

Acetonitrile HPLC grade

Sodium hydroxide

Hydrochloric acid

Milli Q water

Potassium Di hydrogen phosphate

Hydrogen peroxide

Levofloxacin Working standard

Azithromycin Working standard

Instruments Used :

Sartorius electronic balance

LABINDIA pH meter

"FAST – CLEAN" Ultrasonic cleaner

Millipore – solvent filtration unit

Waters e 2695 separations module

4.2METHOD DEVELOPMENT

The objective of this experiment was to optimize the assay method for estimation of Levofloxacin and Azithromycin based on the literature survey. So here the trials mentioned describes how the optimization was done.

TRAIL-1:

Buffer preparation:

Weigh accurately 6.80 g of Potassium Di hydrogen phosphate and 8.709 Di Potassium hydrogen phosphate dissolve it in 1000 ml of Milli-Q water. Adjust the pH to 6.5, filter through 0.45µm nylon membrane filter and degas.

Chromatographic conditions:

Flow rate	:	1.0 ml/min
Column	:	Hypersil C_{18} (150 x 4.6 mm, 5µ)
Detector wave length	:	285 nm
Column temperature	:	35^0c
Injection volume	:	10µl
Run time	:	10 mins
Diluent	:	Mobile phase

Mobile phase: pH 6.5 buffer : Acetonitrile (50:50)

Chromatogram :

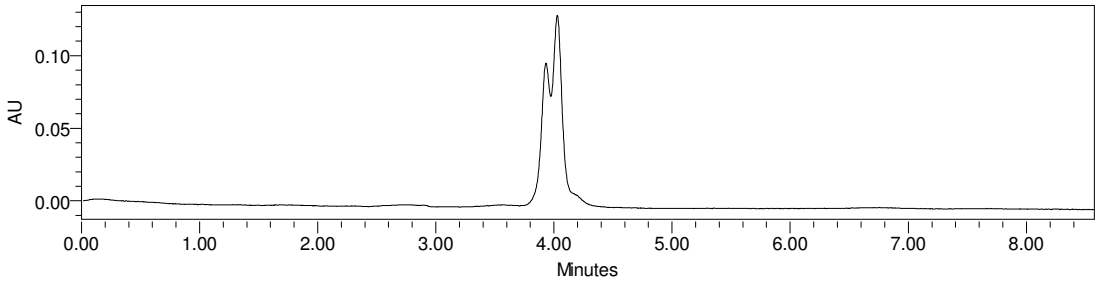


Figure no.4.2.1

Isocratic system:

Table- 4.2.1

S.NO	Name of the peak	Retention time(min)
1	Levofloxacin	3.9
2	Azithromycin	4.1

Result :

It fails system suitability parameters. In second trail – changing of pH

TRIAL-2:

Buffer preparation:

Weigh accurately 6.80 g of Potassium Di hydrogen phosphate& 8.709 g of Di Potassium hydrogen phosphate and dissolve it in 1000 ml of Milli-Q water. Adjust the pH to 5.7, filter through 0.45µm nylon membrane filter and degas.

Chromatographic conditions:

Flow rate	:	1.0 ml/min
Column	:	c_{18} (Hypersil, 4.6 x 250mm, 5µm,)
Detector wave length	:	285 nm
Column temperature	:	35^0c
Injection volume	:	10µl
Run time	:	10 mins
Diluent	:	Mobile phase

Mobile phase: pH 5.7 buffer : acetonitrile (50:50)

Chromatogram :

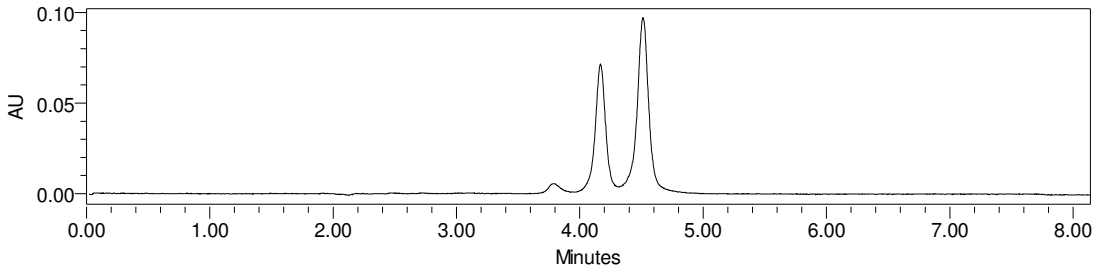


Figure no 4.2.2

Isocratic system :

Table- 4.2.2

S.NO	Name of the peak	Retention time(min)
1.	Levofloxacin	4.3
2	Azithromycin	4.6

Result:

The system suitability parameters are failed. In third trail – changing of pH and mobile phase ratio

TRIAL-3:

Buffer preparation:

Weigh accurately 6.80 g of Potassium Di hydrogen phosphate & 8.709 g of Di Potassium hydrogen phosphate and dissolve it in 1000 ml of Milli-Q water. Adjust the pH to 7.3, filter through 0.45µm nylon membrane filter and degas.

Chromatographic conditions:

Flow rate	:	1.0 ml/min
Column	:	C_{18} (Hypersil 4.6 x 250mm, 5µm,)
Detector wave length	:	285 nm
Column temperature	:	35^0c
Injection volume	:	10µl
Run time	:	9mins
Diluent	:	Mobile phase

Mobile phase: pH 7.3 buffer : Acetinitrile (70:30)

Chromatogram :

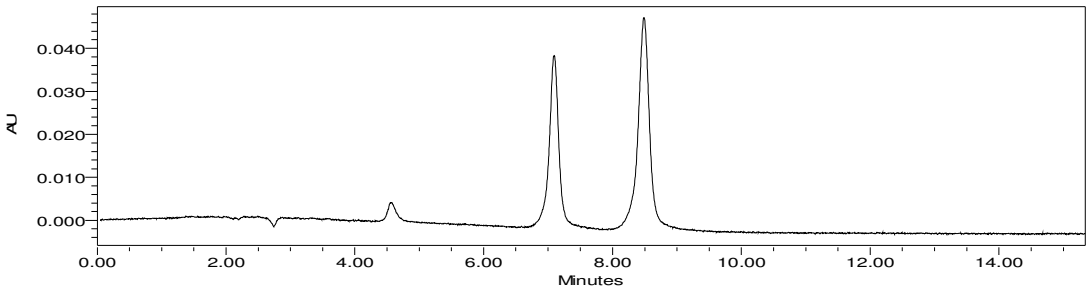


Figure no 4.2.3

Isocratic programme:

Table – 4.2.3

S.NO	Name of the peak	Retention time(min)
1	Levofloxacin	7.1
2	Azithromycin	8.5

Result:

The peak height and shape was good and system suitability parameters are failed.in fourth trail changing of mobile phase ratio.

TRIAL-4:

Buffer preparation:

Weigh accurately 6.80 g of Potassium Di hydrogen phosphate & 8.709 g of Di Potassium hydrogen phosphate and dissolve it in 1000 ml of Milli-Q water. Adjust the pH to 7.3, filter through 0.45µm nylon membrane filter and degas.

Chromatographic conditions:

Flow rate	:	1.0 ml/min
Column	:	C_{18} (Hypersil 4.6 x 250mm, 5µm,)
Detector wave length	:	285 nm
Column temperature	:	35^0c
Injection volume	:	10µl
Run time	:	9mins
Diluent	:	Mobile phase

Mobile phase: pH 7.3 buffer : Acetinitrile (60:40)

Chromatogram:

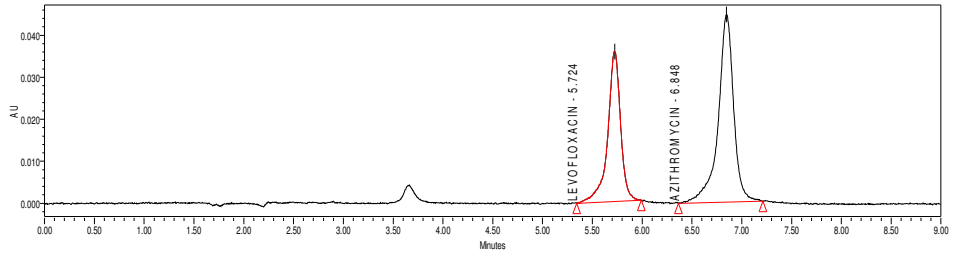


Figure no. 4.2.4

Isocratic programme:

Table – 4.2.4

S.NO	Name of the peak	Retention time(min)
1	Levofloxacin	5.7
2	Azithromycin	6.8

Result:

The peak height and shape was good and system suitability parameters are passed.

OPTIMIZED METHOD:

Buffer preparation:

Weigh accurately 6.80 g of Potassium Di hydrogen phosphate & 8.709 g of Di Potassium hydrogen phosphate and dissolve it in 1000 ml of Milli-Q water. Adjust the pH to 7.3filter through 0.45µm nylon membrane filter and degas.

Chromatographic conditions:

Flow rate : 1.0 ml/min

Column : C_{18} (hypersil 4.6 x 250mm, 5µm,)

Detector wave length : 285 nm

Column temperature : 35^0c

Injection volume : 10µl

Run time : 9mins

Diluent : Mobile phase.

Mobile phase: pH 7.3 buffer : Acetinitrile (60:40)

Chromatogram:

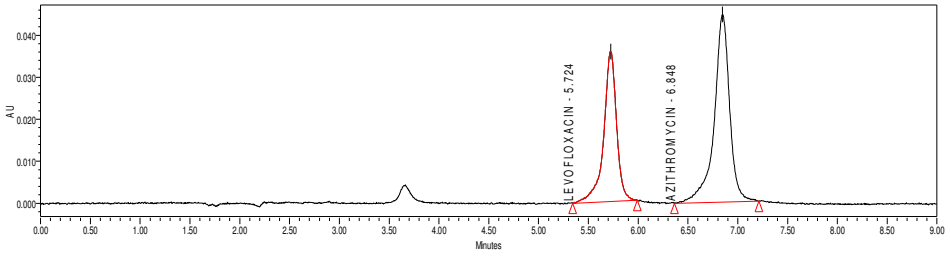


Figure no. 4.2.5

Table – 4.2.5

	Name	Retention Time	Area	% Area	Height	USP Resolution	s/n	USP Tailing	USP Plate Count
1	LEVO	5.721	317720	40	35715	4.712	86.962	0.835	11519
2	AZIT	6.848	464547	60	44659		108.082	0.874	11695

Result:

The system suiability parameters have been passed for above method. So we can use it as optimized method.

ASSAY OF FORMULATION BY DEVELOPED METHOD:

Preparation of buffer:

Weigh accurately 6.80 g of Potassium Di hydrogen phosphate & 8.709 g of Di Potassium hydrogen phosphate and dissolve it in 1000 ml of Milli-Q water. Adjust the pH to 7.3.

Mobile phase preparation: Mix up 600ml of Buffer and 400ml of Acetonitrile anddegass to remove air bubbles.

Standard preparation:

Transfer about 10 mg of Levofloxacin working standard and 10 mg of Azithromycinin to 100ml volumetric flask, dissolve and dilute to volume with water and mixed.

Test preparation:

For estimating the tablet dosage form randomly select 5 tablets(LEVOMAC-AZ) from a batch and make it powder, and weigh accurately 2760 mg of powder(equivalent to 1000 mg Levofloxacin and 1000 mg of Azithromycin) and transfer to 100ml volumetric flask. Then add 70 ml of water shake the flask on a rotator shaker for 30 min and sonicate for 15 min with intermediate shaking. Keep the solution on a rotatory shaker for 30 min at 200 rpm. Centrifuge the portion of above solution at 4000 rpm for 5 min. Pipette out 1 ml of above clear solution and transfer it to 100 ml volumetric flask and make up the volume with water.

Assay formula:

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \text{average weight of tablet}$$

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

Acceptance criteria: The limit of assay is in between the 98% - 102%

Table no 4.2.6 showing Assay Results of Levofloxacin&Azithromycin:

DRUG	PEAK AREA	ASSAY %
Levofloxacin	321745	99%
Azithromycin	471405	99%

Chromatogram showing for standard solution :

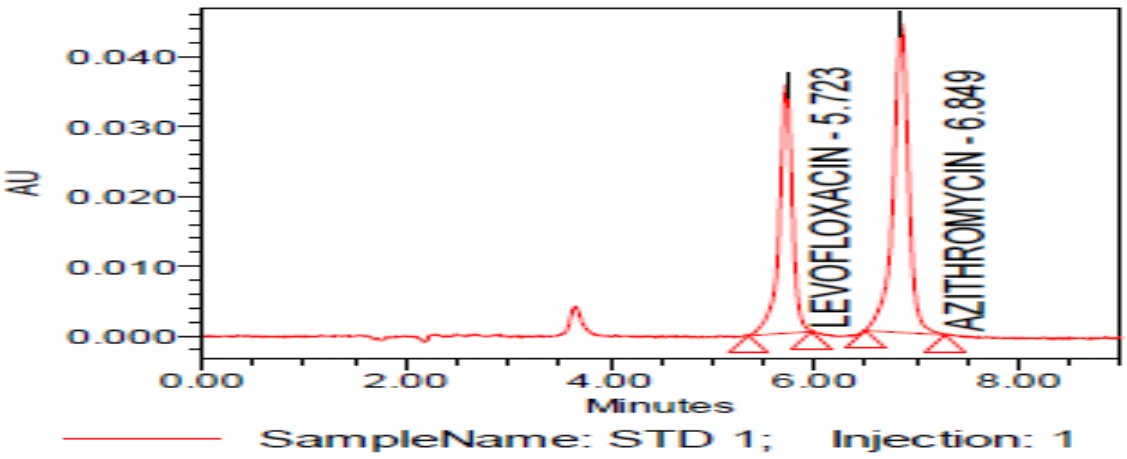


Figure no 4.2.6

Chromatogram showing for test solution:

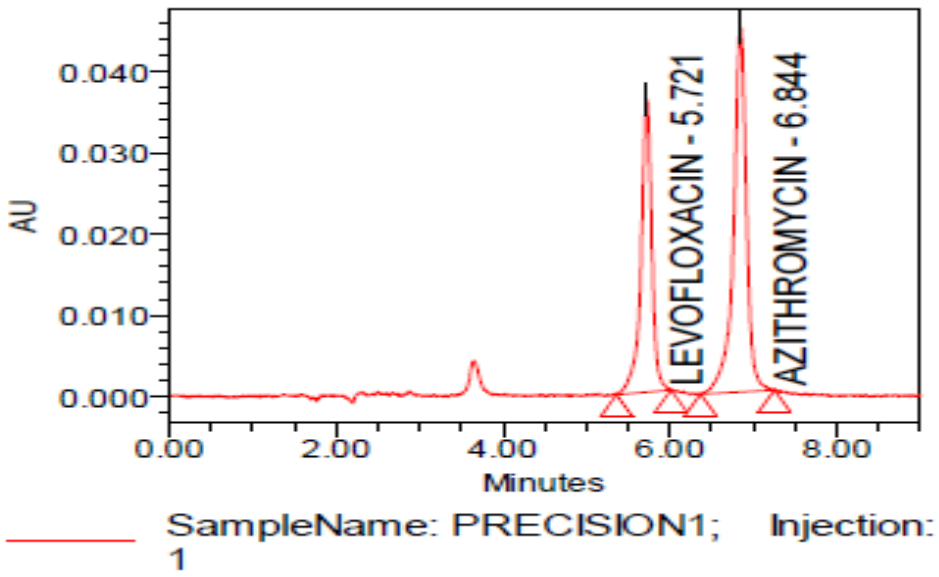
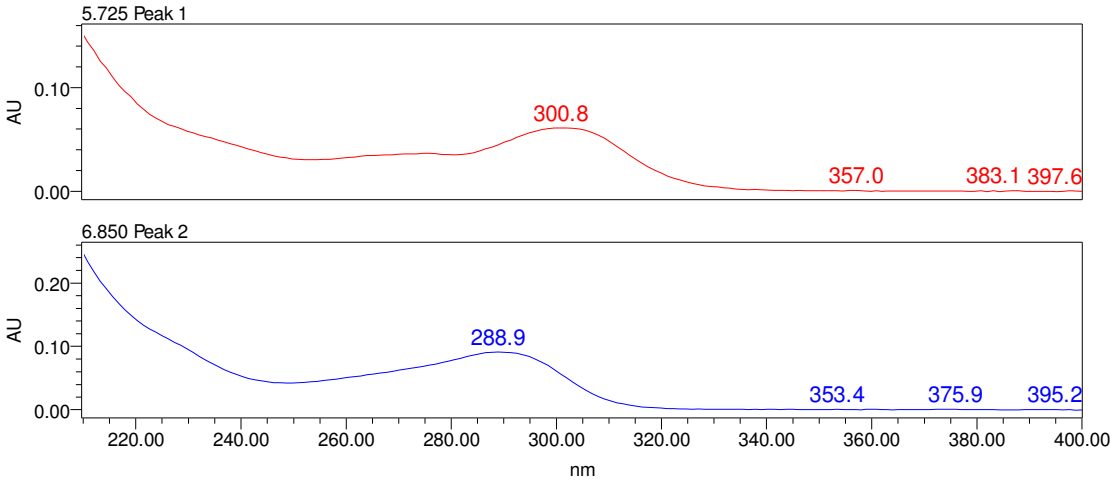


Figure no 4.2.7

UV- spectrum of Levofloxacine and Azithromycin:



4.3 METHOD VALIDATION

After the method development the method was validated in terms of parameters like Precision, Accuracy Specificity, Linearity, and Robustness,system suitability, stability etc.

OPTIMIZED METHOD:

Buffer preparation: Weigh 6.80 & 8.70 gm of KH_2PO_4& K_2HPO_4 in 1000ml of water and adjust the pH to 7.30with phosphate buffer and filter through 0.45µm nylon membrane filter and degas.

Chromatographic conditions:

Flow rate	:	1.0 ml/min
Column	:	C_{18} (Hypersil 4.6 x 250mm, 5µm,)
Detector wave length	:	285 nm
Column temperature	:	35^0c
Injection volume	:	10µl
Run time	:	9 mins
Diluent	:	Mobile phase

Mobile phase: pH 7.30 buffer :Acetinitrile (60:40)

Isocratic programme:

Table no: 4.3.1

s.no	**Name of the peak**	**Retention time**
1	**Levofloxacin**	**5.710**
2	**Azithromycin**	**6.848**

4.3.1 SYSTEM SUITABILITY :

A Standard solution of working standard was prepared as per procedure and was injected six times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from six replicate injections.

Acceptance criteria:

The % RSD for the retention times of principal peak from 6 replicate injections of each Standard solution should be not more than 2.0 %

The number of theoretical plates (N) should be not less than 2000.

The resolution should be more than 2

THEORETICAL PLATES:

A standard solution of 10µlof Levofloxacin and Azithromycinwas injected, then the system suitability parameters were calculated from the chromatogram.

Calculation of number of theoretical plates

$N/L = 5.54 (V_e / W_{½})^2$

Where:N=Number of theoretical plates

L=Length of column (m)

V_e=Elution volume of an unretained non interactive molecule

H=Height of peak

$W_{1/2}$ =Width of the peak at half peak height

RESOLUTION:

Resolution of the method was calculated by the formula.

$R = 2 (t_2 – t_1)/ (w_2 + w_1)$

Where, $\mathbf{t_1}$ and $\mathbf{t_2}$are Retention times of the components.

w1 + w2 are corresponding width at the bases of the peak of components.

Method:

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Levofloxacin and 10 mg of Azithromycin working standards into a 100 ml clean dry volumetric flask add about 70 ml of Diluent and sonicate for 30 mts to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Table No:4.3.1.1System suitability Parameters

System Suitability Parameters	**Levofloxacin**	**Azithromycin**
Resolution	4.706	
Tailing Factor	0.835	0.874
Number of theoretical Plates	11519	11695
Retention time	5.724	6.848

Chromatogram showing system suitability testing of Standard Solution -1 ofLevofloxacin and Azithromycin:

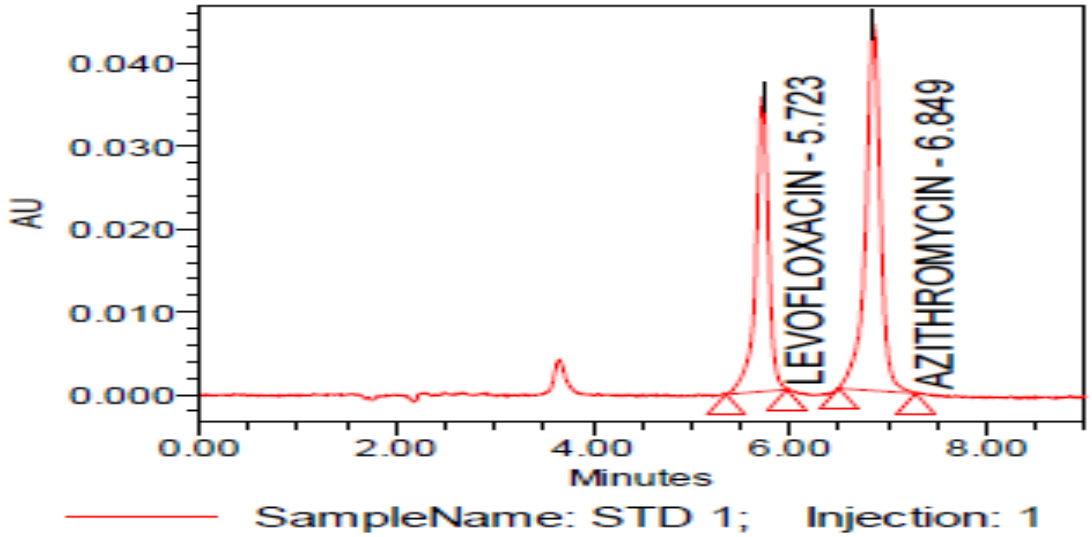


Figure no: 4.3.1.1

Chromatogram showing system suitability testing of Standard Solution2 -1 ofLevofloxacin and Azithromycin:

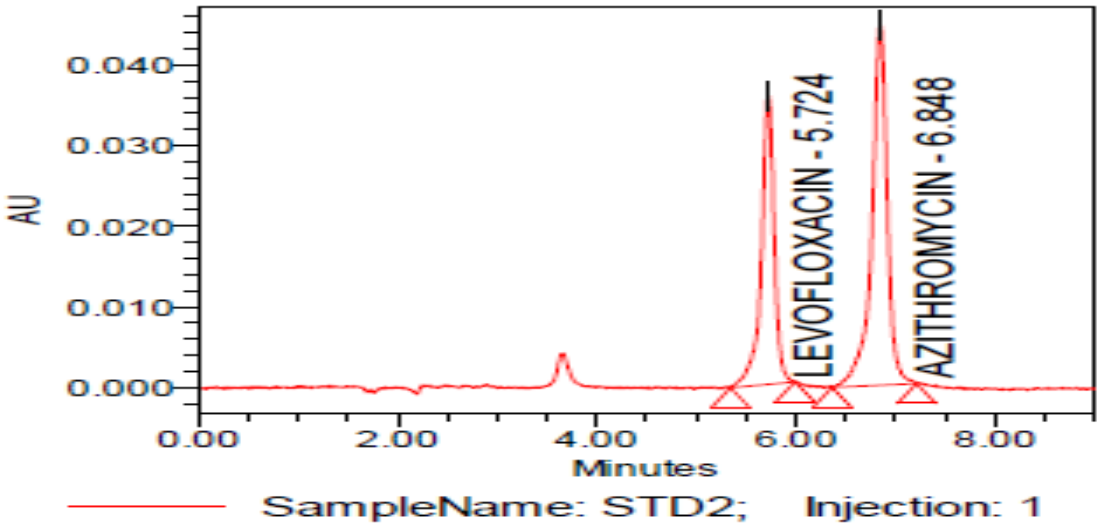


Figure no: 4.3.1.2

Chromatogram showing system suitability testing of Standard Solution2 -2 ofLevofloxacin and Azithromycin:

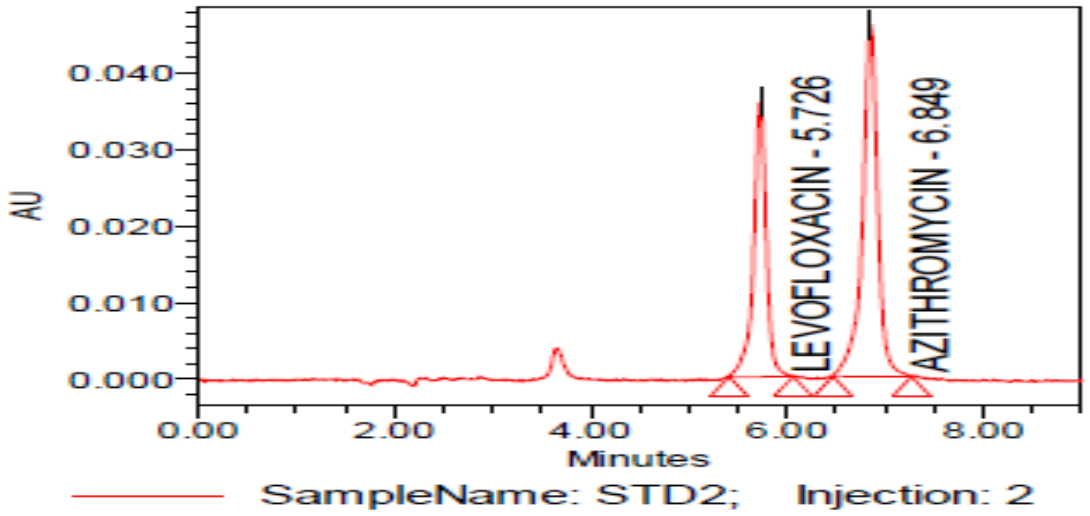


Figure no. 4.3.1.3

Chromatogram showing system suitability testing of Standard Solution2 -3 ofLevofloxacin and Azithromycin:

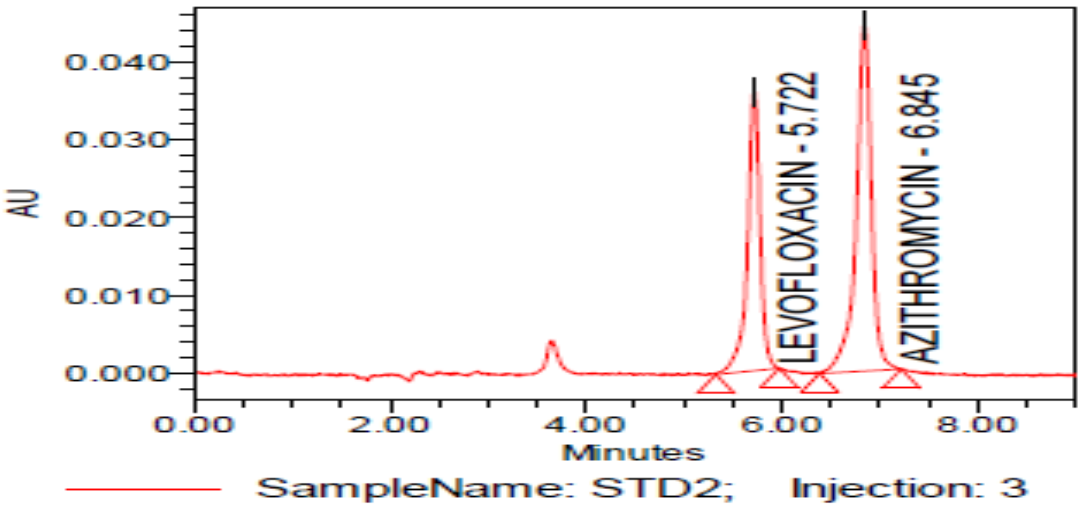


Figure no: 4.3.1.4

Chromatogram showing system suitability testing of Standard Solution2 -4 ofLevofloxacin and Azithromycin:

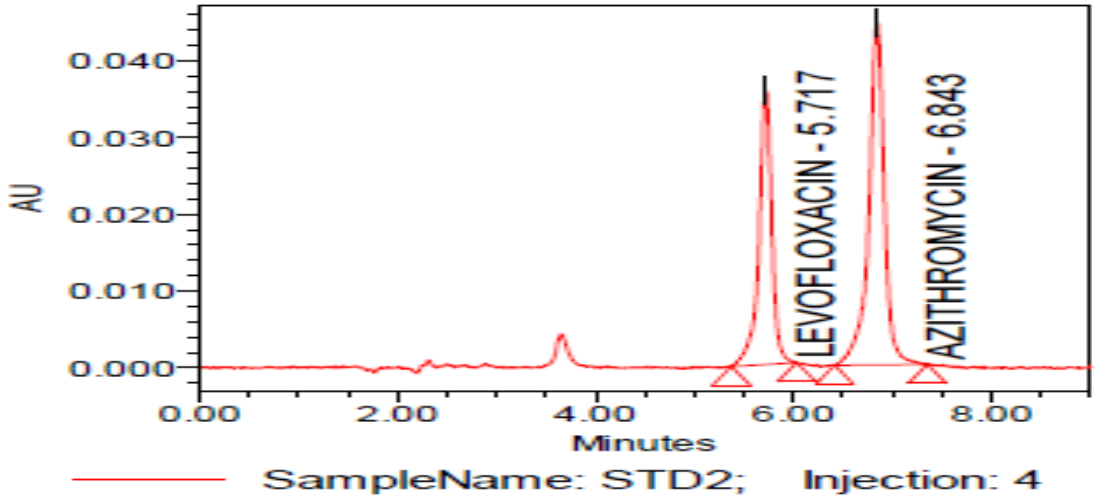


Figure no: 4.3.1.5

Chromatogram showing system suitability testing of Standard Solution2 -5 ofLevofloxacin and Azithromycin:

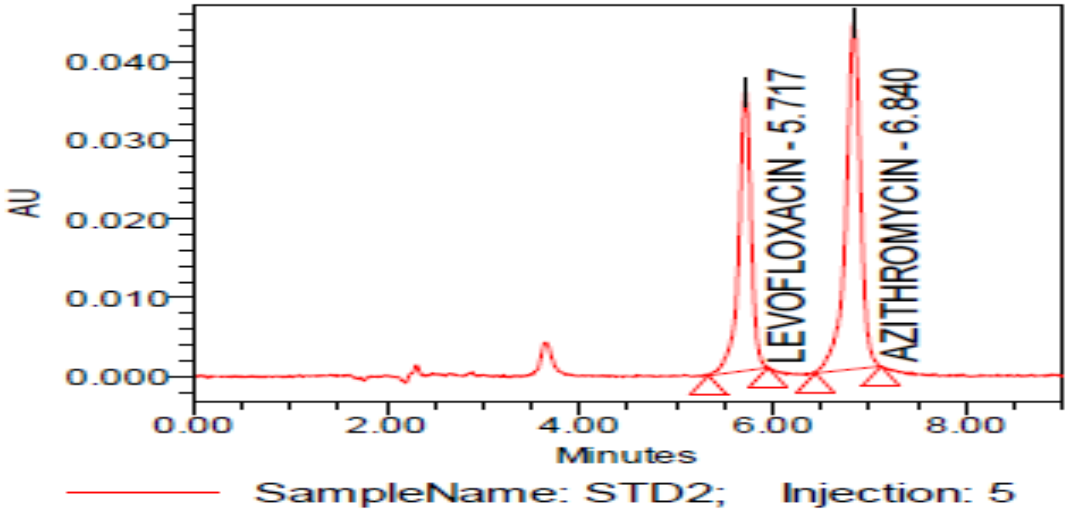


Figure 4.3.1.6

4.3.2 ACCURACY:

Accuracy expresses the closeness of agreement between the value, which was accepted either as conventional true value or and accepted reference value (International standard e.g. pharmaceutical standard) and the value found (mean value) obtained by applying the test procedure a number of times.

To study reliability, suitability and accuracy of the method, recovery studies were carried out, by adding a known quantity of the standard to the pre analysed sample and recovery study were done. The recovery was carried out at 50%, 100%, 100% level and the contents were determined from respective chromatogram .From the results obtained we conclude that the method was accurate.

Preparation of Standard stock solution:

Accurately weigh and transfer 10 mg ofLevofloxacin and 10 mg of Azithromycin working standards into a 100 ml clean dry volumetric flask add about 70 ml of Diluent and sonicate for 30 mts to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately weigh and transfer 5 mg of Levofloxacin and 5 mg of Azithromycinworking standard into a 100 ml clean dry volumetric flask add about 70 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock Solution).

Repeat the above procedure and Prepare another 5 solutions.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately weigh and transfer 10 mg of Levofloxacin and 10 mg of Azithromycinworking standards into a 100 ml clean dry volumetric flask add about 70 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.(Stock solution)

Repeat the above procedure and Prepare another 2 solutions.

For preparation of 150% solution (With respect to target Assay concentration):

Accurately weigh and transfer 15 mg of Levofloxacin and 15 mg of Azithromycinworking standards into a 100 ml clean dry volumetric flask add about 70 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.(Stock solution).

Repeat the above procedure and Prepare another 5 solutions

Procedure:

Inject the 10µl standard solution, Accuracy -50% for 6 times, Accuracy -100% for 3 times and Accuracy -100% for 6 times.

Calculate the amount present and amount added for the Levofloxacin and Azithromycin

formula $= \frac{amount\ recovered}{amount\ added} \quad x \ 100$

Acceptance criteria:

The mean % recovery of theLevofloxacin and Azithromycinat each level should be not less than 97.0% and not more than 103.0%.

Table no: 4.3.2 Showing results for % recovery studies for Levofloxacin and Azithromycin:

Spike level	Inj. no	LEVO Peak Area	LEVO µgm/ml added	LEVO µgm/ml found	% recovery	AZI Peak area	AZI µgm/ml added	AZI µgm/ml found	% recovery	% MEAN	
										LEV	AZI
50%	1	152252	48.065	46.95	98	228541	47.580	47.01	99	100	99
	2	154749	48.209	47.92	99	226084	47.722	46.51	97		
	3	159354	48.245	49.14	102	228450	47.757	46.99	98		
	4	156581	47.993	48.29	101	232162	47.509	47.76	101		
	5	155679	48.029	48.01	100	230592	47.544	47.43	100		
	6	155469	48.037	47.94	100	235627	47.551	48.47	102		
100%	1	317762	99.056	97.99	99	469659	98.000	96.61	99	99	99
	2	317787	99.077	98.00	99	469606	98.007	96.60	99		
	3	321825	99.541	99.25	100	477889	98.004	98.30	100		
150%	1	489645	147.424	151.00	102	718447	145.935	147.79	101	102	101
	2	491204	147.426	151.48	103	727905	145.935	149.73	103		
	3	480293	147.435	148.11	100	726781	145.945	149.50	102		
	4	498550	147.438	153.74	102	717654	145.939	147.62	101		
	5	485500	147.442	149.72	102	710231	145.953	146.10	100		
	6	488057	147.435	150.51	102	698460	145.945	143.67	98		

Chromatogram for accuracy 50% - 1 solution:

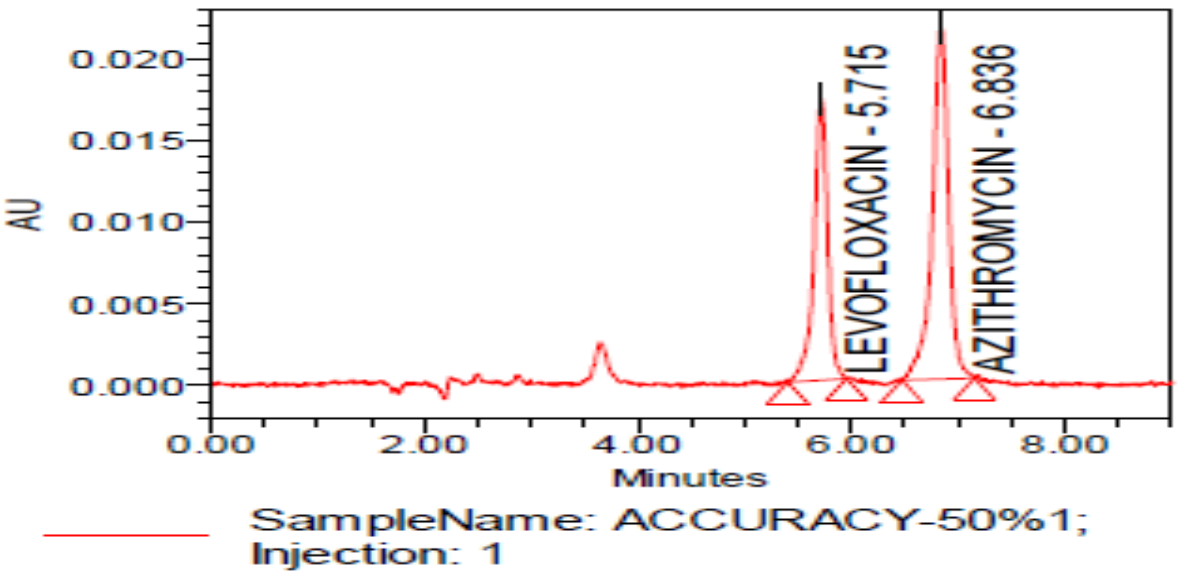


Figure no: 4.3.2.1

Chromatogram for accuracy 50% - 2 solution:

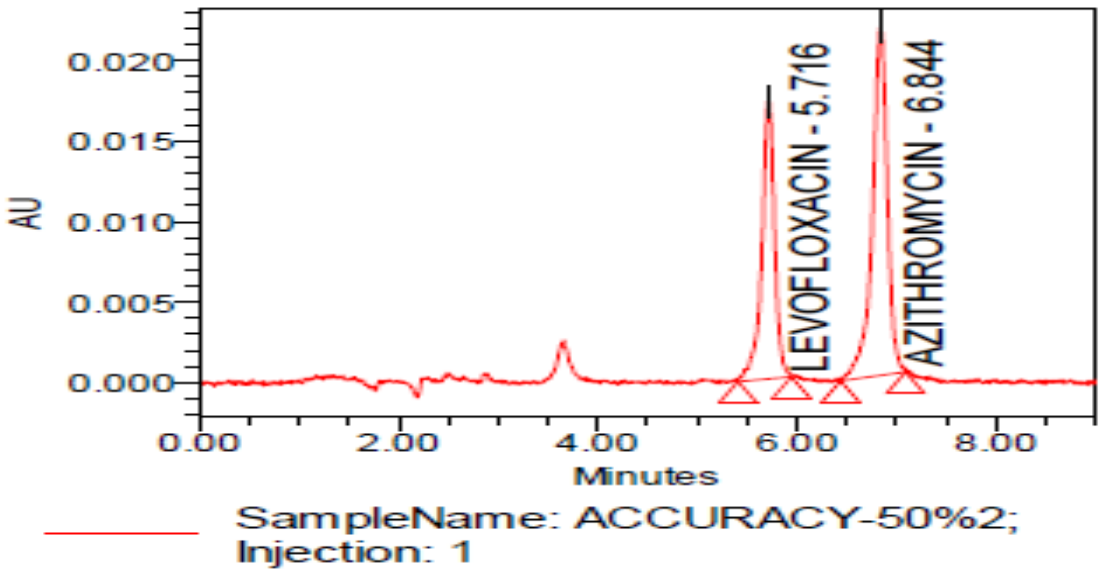


Figure no: 4.3.2.2

Chromatogram for accuracy 50% - 3solution:

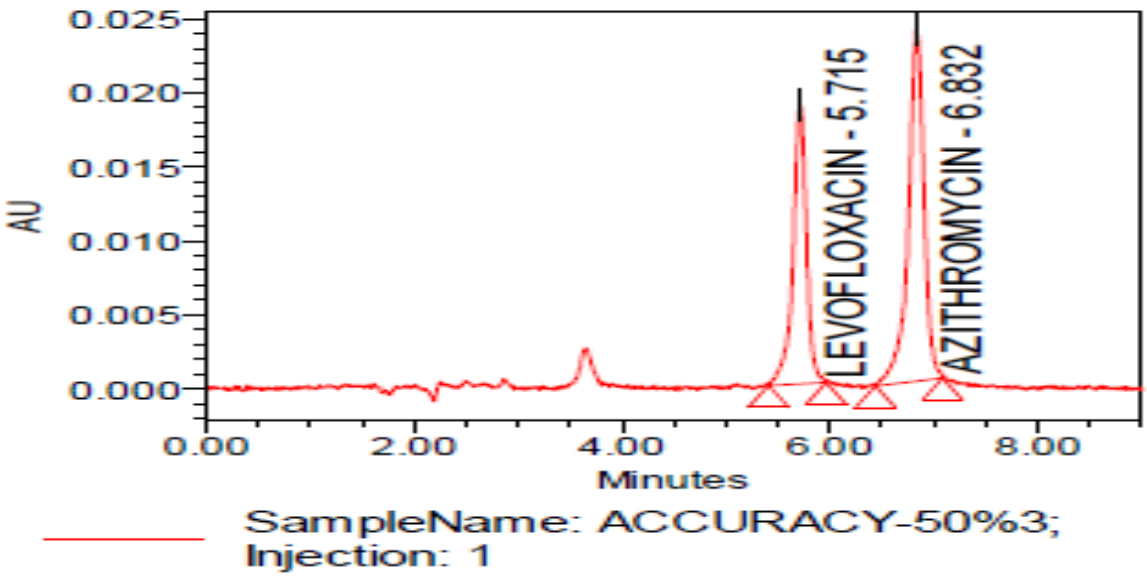


Figure no: 4.3.2.3

Chromatogram for accuracy 50% - 4 solution:

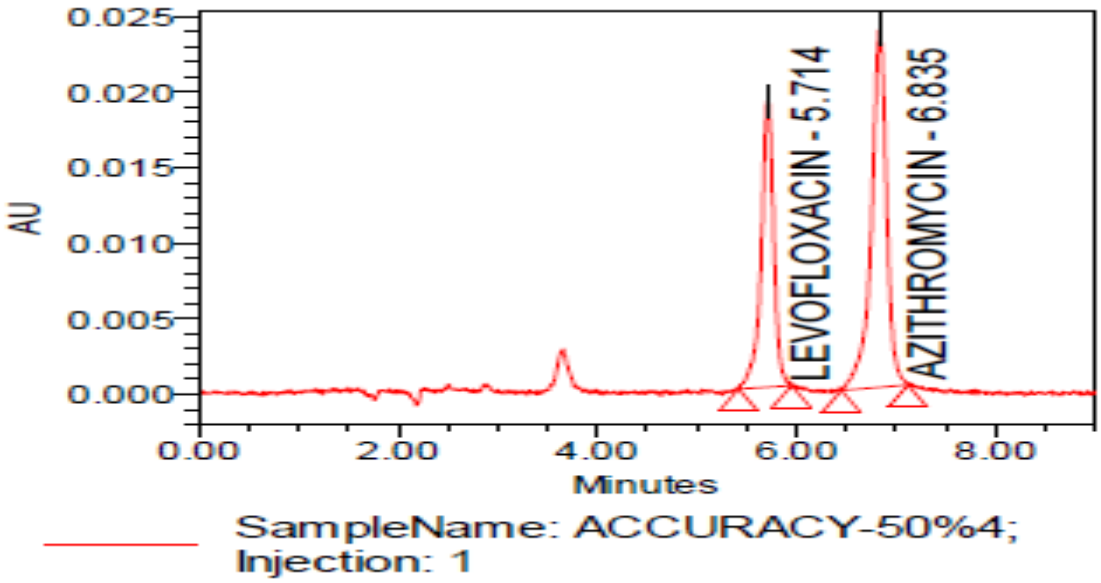


Figure no: 4.3.2.4

Chromatogram for accuracy 50% - 5 solution:

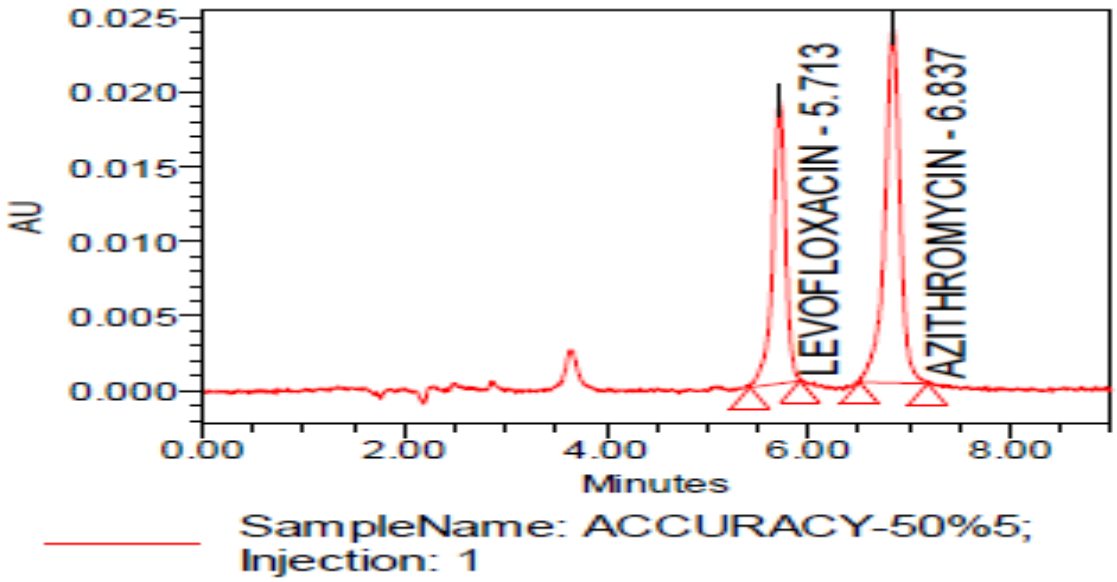


Figure no: 4.3.2.5

Chromatogram for accuracy 50% - 6 solution:

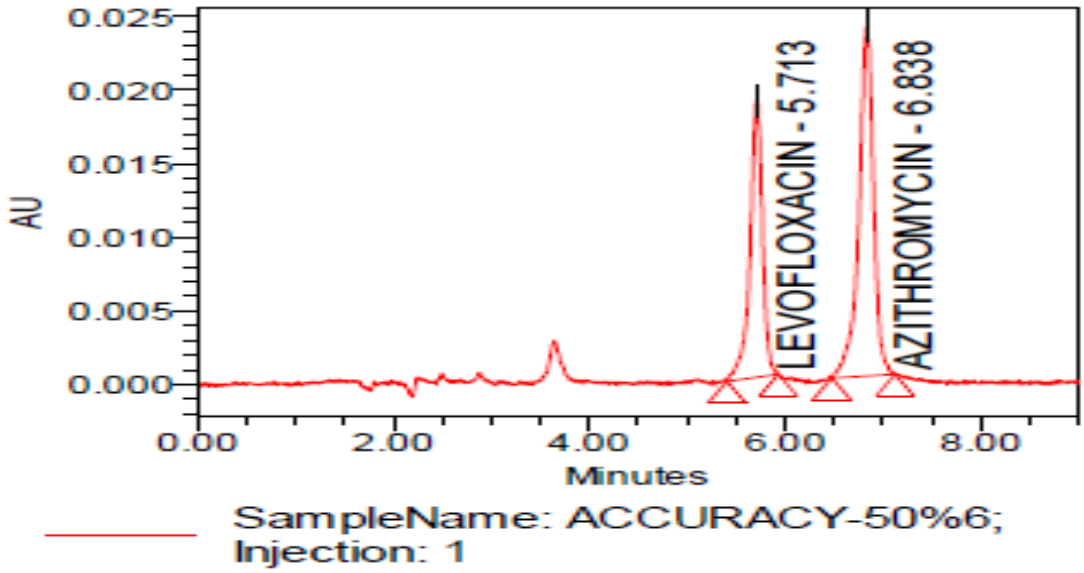


Figure no: 4.3.2.6

Chromatogram for accuracy 100% - 1 solution:

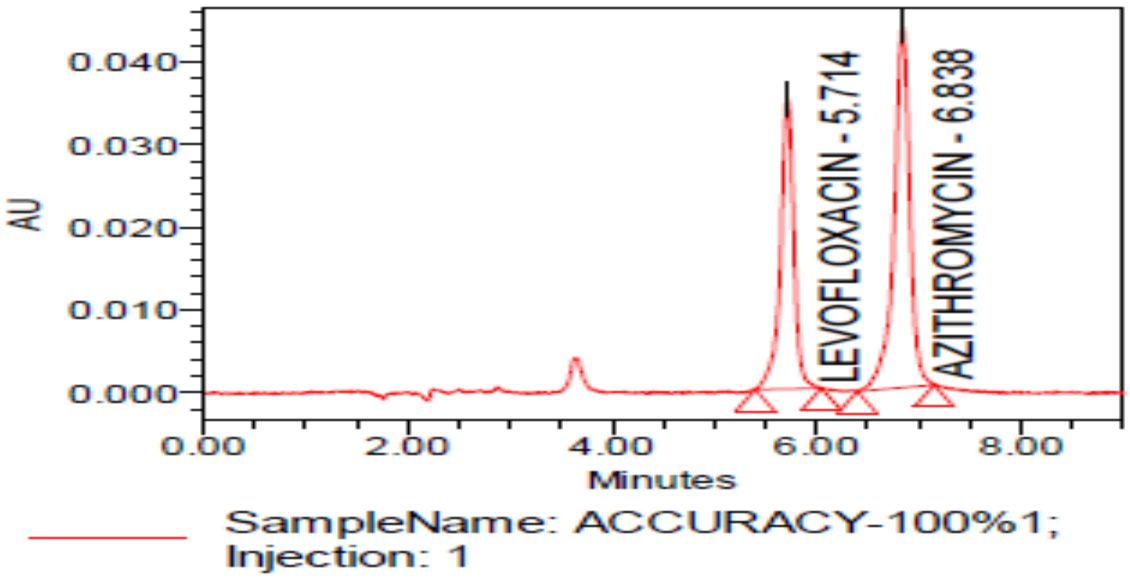


Figure no: 4.3.2.7

Chromatogram for accuracy 100% - 2 solution:

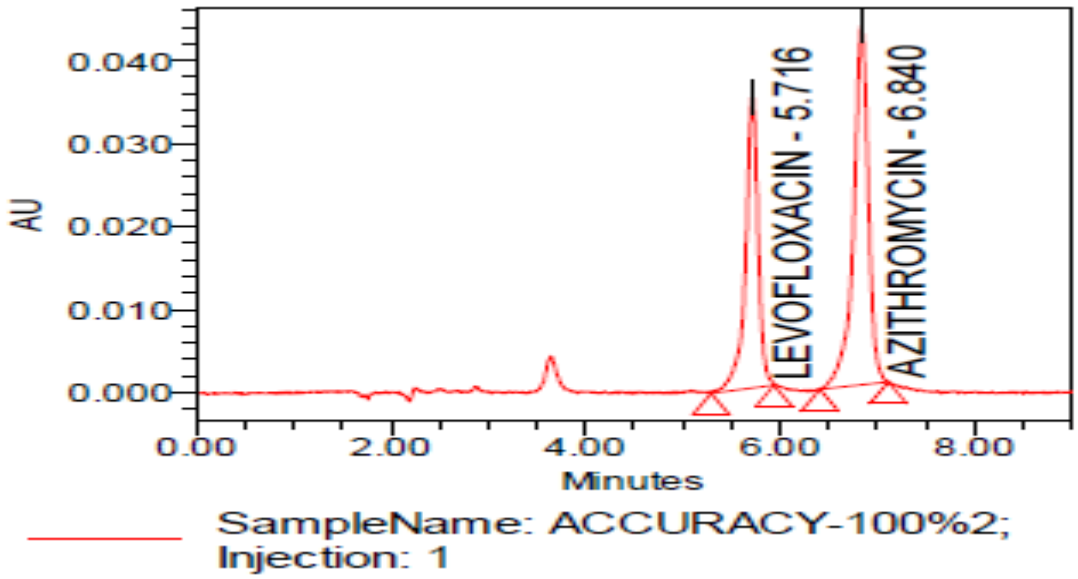


Figure no: 4.3.2.8

Chromatogram for accuracy100% - 3 solution:

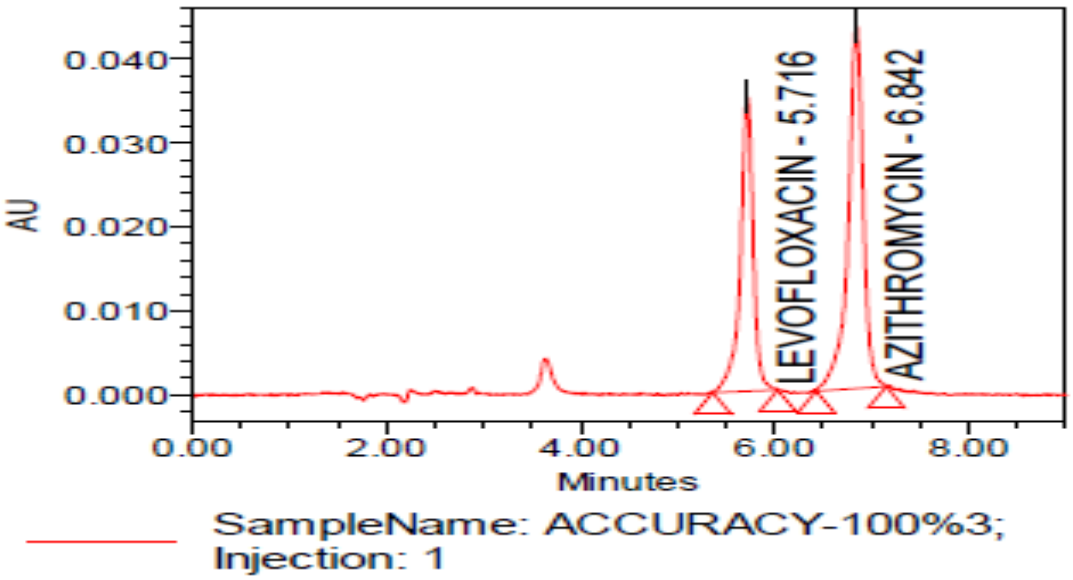


Figure no: 4.3.2.9

Chromatogram for accuracy150% - 1 solution:

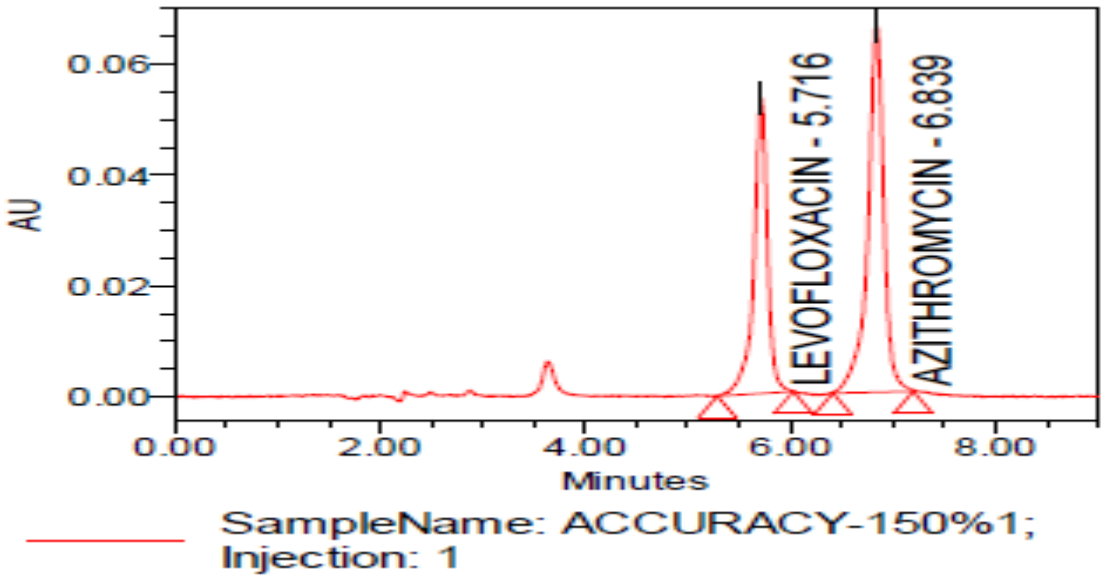


Figure no: 4.3.2.10

Chromatogram for accuracy150% - 2 solution:

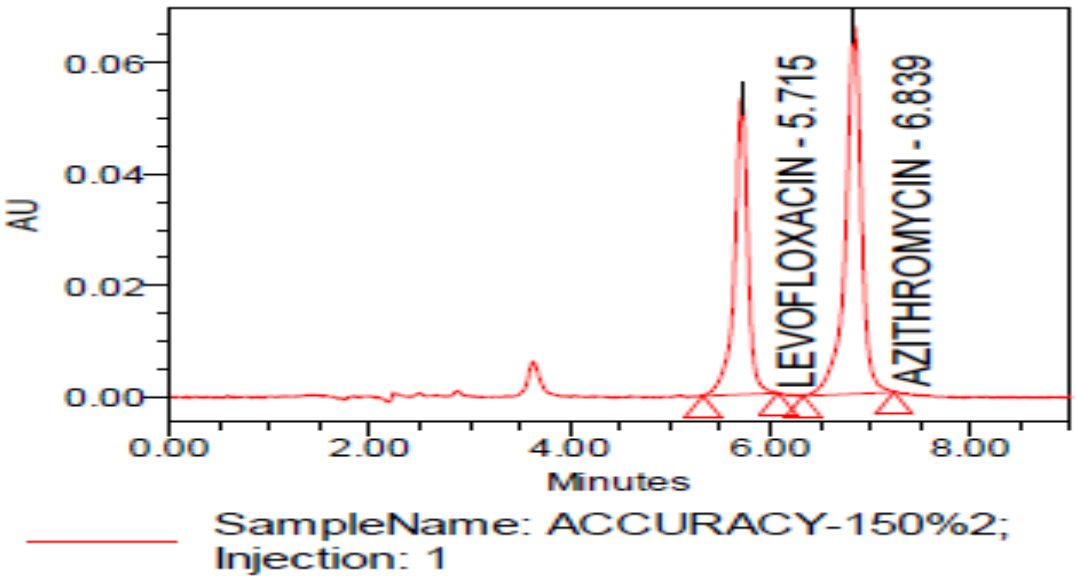


Figure no: 4.3.2.11

Chromatogram for accuracy150% - 3 solution:

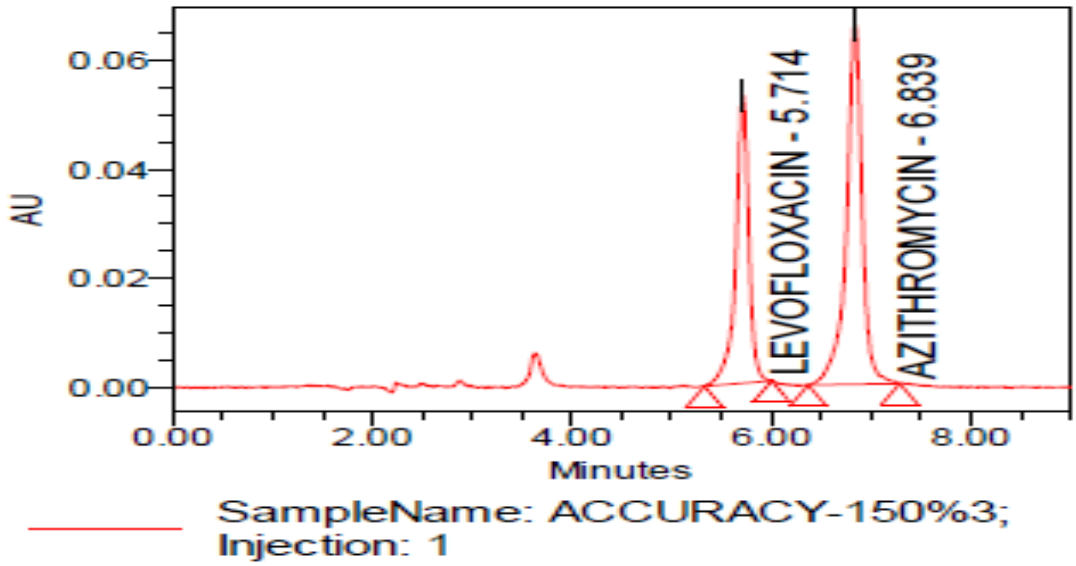


Figure no: 4.3.2.12

Chromatogram for accuracy150% - 4 solution:

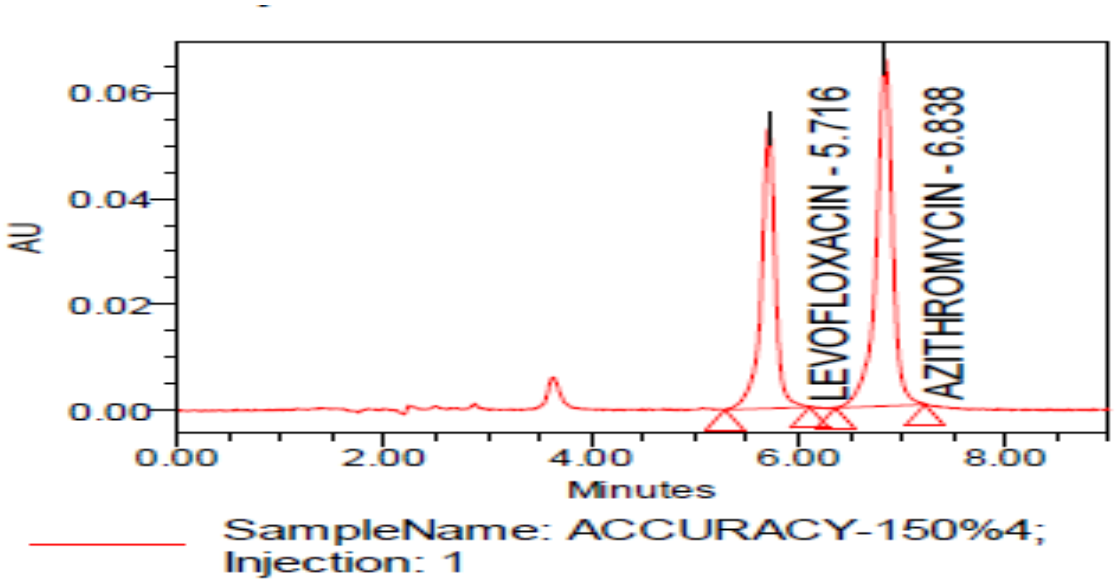


Figure no: 4.3.2.13

Chromatogram for accuracy150% - 5 solution:

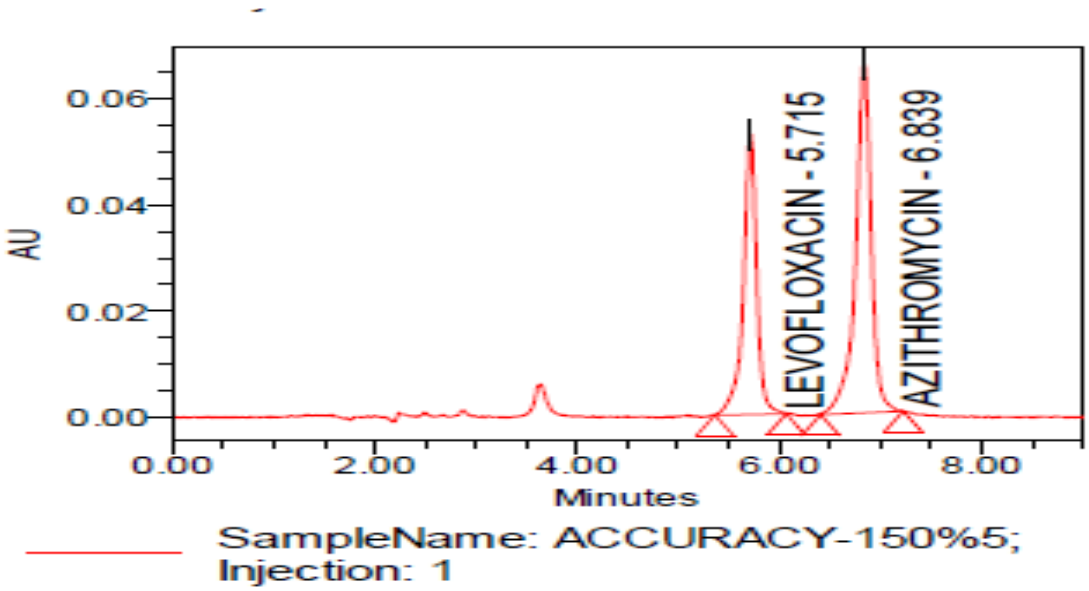


Figure no: 4.3.2.14

Chromatogram for accuracy150% - 6 solution:

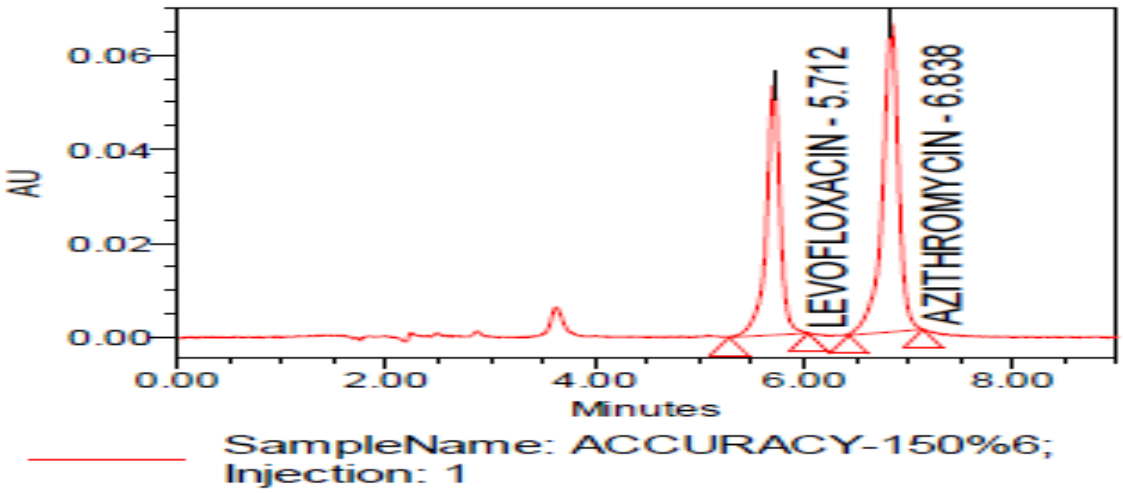


Figure no: 4.3.2.15

Chromatogram showing for the Accuracy of Levofloxacin and Azithroycin:

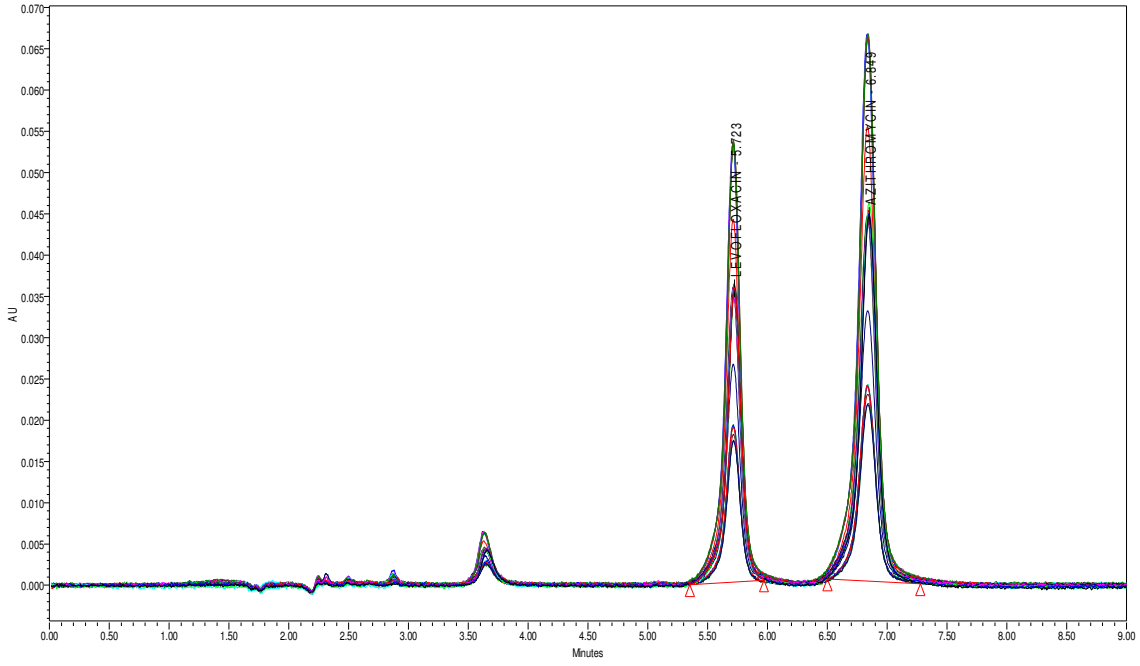


Figure no: 4.3.2.16

4.3.3 PRECISION:

The Precision of test method was done by performing assay on five replicate determination of sample preparation at test concentration level (as per method of analysis) and calculated relative standard deviation of assay results.

5 injections from standard solutions were injected and the peak areas were obtained and %RSD was calculated. System precision and method precision were determined.

5 same concentrations of standard solution are prepared with mobile phase. 10µL of the standard solutions were injected and chromatograms were recorded and the average % RSD values of the results were calculated.

Standard Deviation

$$S.D = \sqrt{\frac{\sum (x - x_i)^2}{n-1}}$$

Where, x=Sample,

x_i=Mean value of samples.

n=number of samples.

Coefficient of variance / Relative standard deviation

$$C.V = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

Procedure:

2760 mg of sample into 100 ml of volumetric flask and add 30 ml of water and sonicateto dissolve and make up with the diluents.

Transfer the above solution 1 ml into 100 ml volumetric flask and dilute the volume with water.

Table no. 4.3.3.1System Precision Levofloxacin and Azithromycin:

Sample No	**Area of Levofloxacin (mv)**	**Area of Azithromycin(mv)**
1	**321374**	**482012**
2	**321580**	**495284**
3	**322171**	**482143**
4	**323889**	**486140**
5	**316130**	**482704**
Mean	**321029**	**485657**
Std .dev	**2912**	**5641**
%RSD	**0.9**	**1.2**

Table no. 4.3.3.2Method Precision of Levofloxacin and Azithromycin:

Sample No	Area of Levofloxacin (mv)	Area of Azithromycin(mv)
1	324952	483236
2	319273	474541
3	329684	471291
4	322242	455970
5	312092	474530
6	322226	468864
Mean	321745	471405
Std .dev	5884	8992
%RSD	1.8	1.9

Acceptance criteria:

% Relative standard deviation of results should not be more than 2.0 %.

Intermediate precision: (Intra day& Inter day studies)

Intra day studies: It was performed by applying the proposed method on same sample of solution on different days.

Table 4.3.3.3Intra day Studies

Day	Peak area of Levofloxacin	Mean	%RSD	Peak area of Azithromycin	Mean	% RSD
Day 1	321745	321726	0.9	482012	482408	1.2
	321699			482143		
	321754			482704		
Day 2	319725	319356	1.05	471945	471524	1.3
	319122			471452		
	319421			471254		

Interdaytudies:: It was performed by applying the proposed method on same sample of solution on same day at 2 hrs interval.

Table 4.3.3.4 Interday Studies

Time	Area of Levofloxacin	Mean	% RSD	Area of Azithromycin	Mean	% RSD
0^{th} hour	321374	321754	0.90	483149	482971	1.20
	321580			482724		
	322171			482965		
3^{rd} hour	322889	322025	0.91	482912	482854	1.20
	321029			482841		
	321564			482754		
6^{th} hour	321523	321839	0.90	481452	481685	1.26
	321421			481921		
	322014			481854		

Chromatogram showing for precision 1:

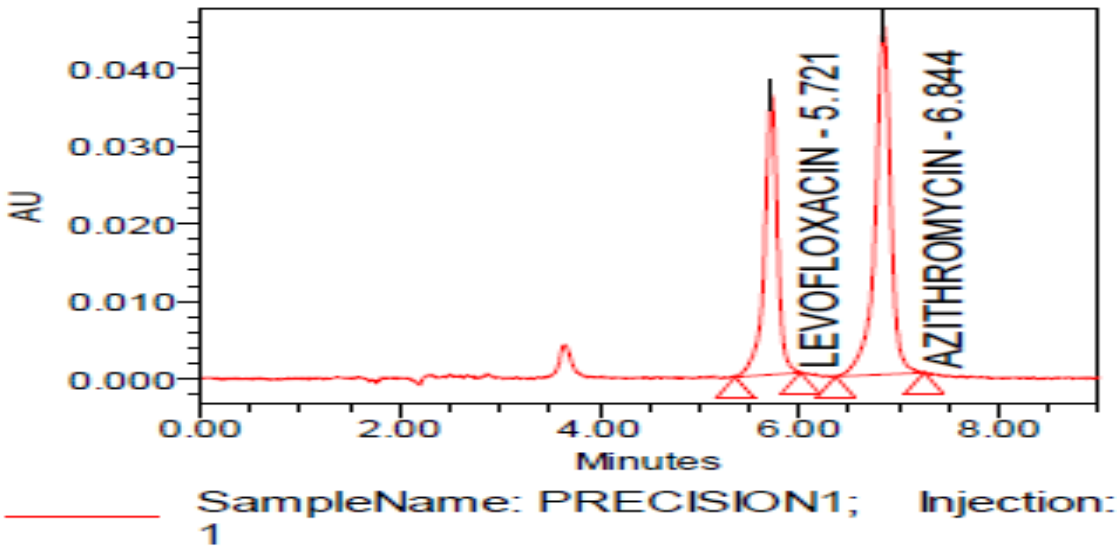


Figure no: 4.3.3.1

Chromatogram showing for precision2:

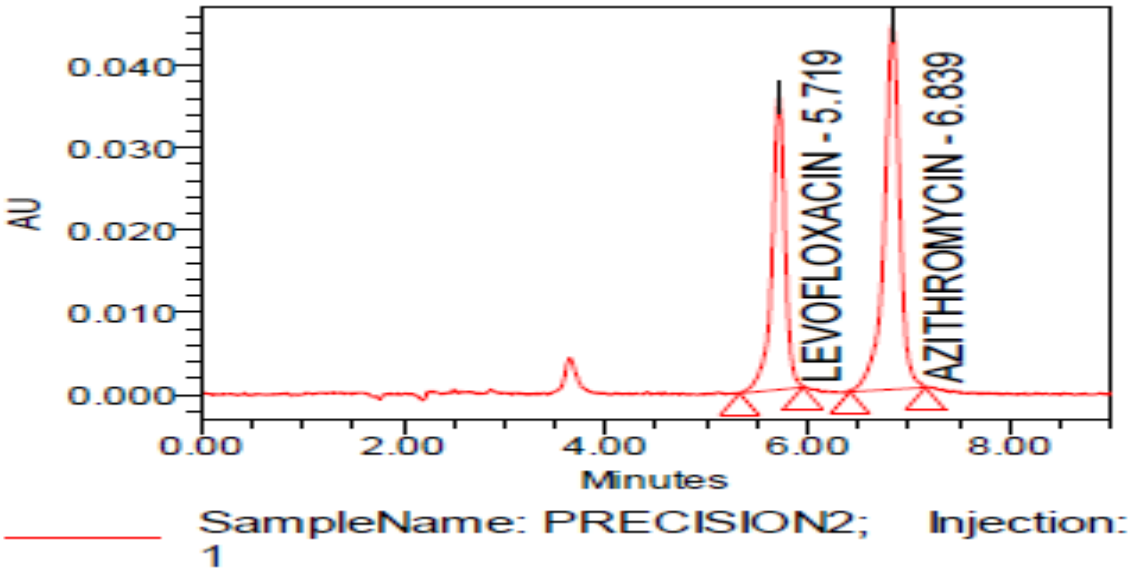


Figure no: 4.3.3.2

Chromatogram showing for precision3:

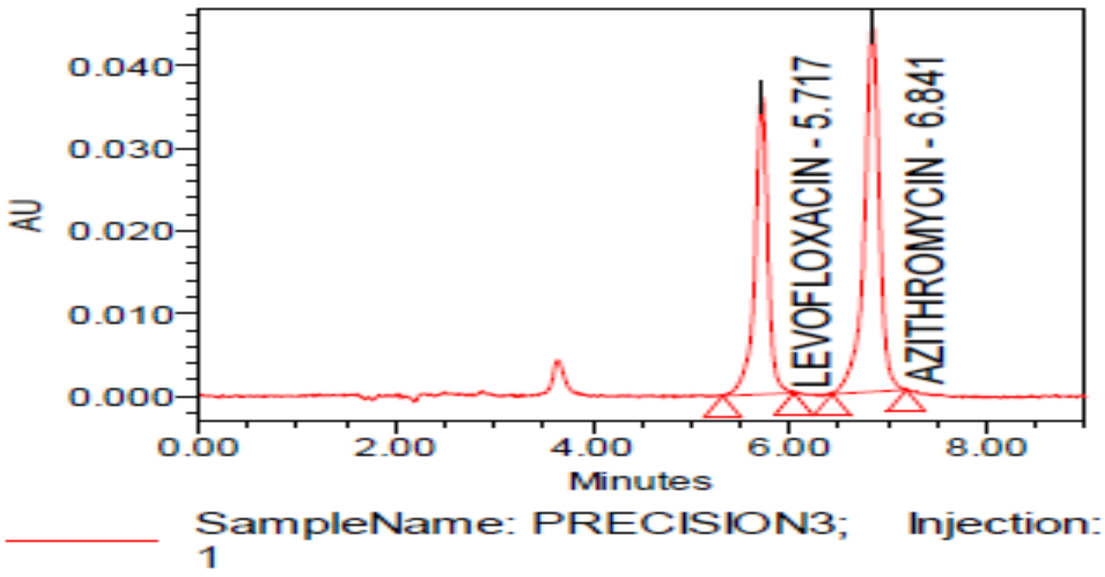


Figure no: 4.3.3.3

Chromatogram showing for precision 4:

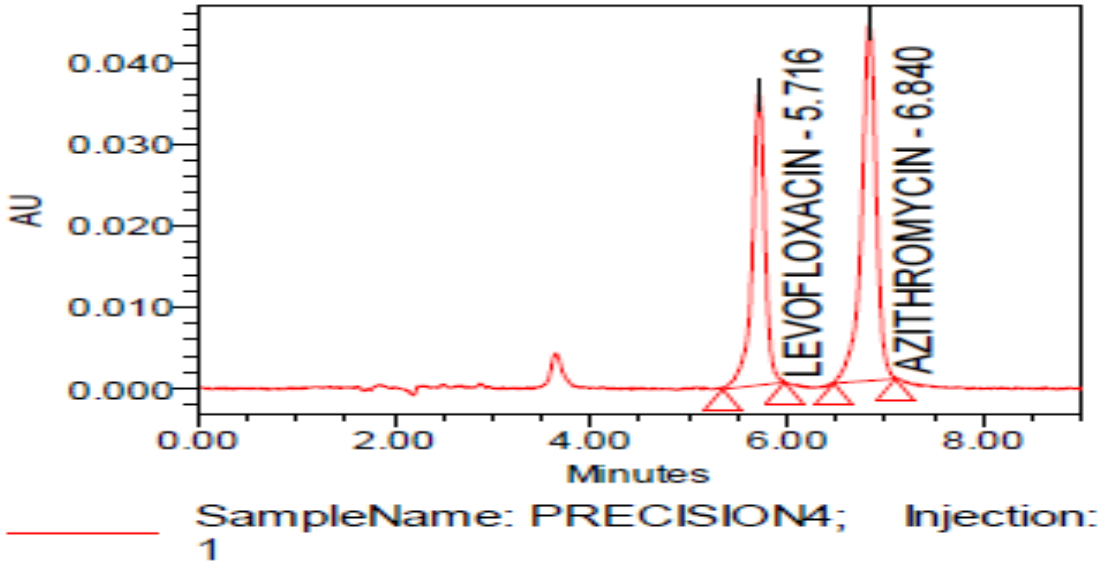


Figure no: 4.3.3.4

Chromatogram showing for precision 5:

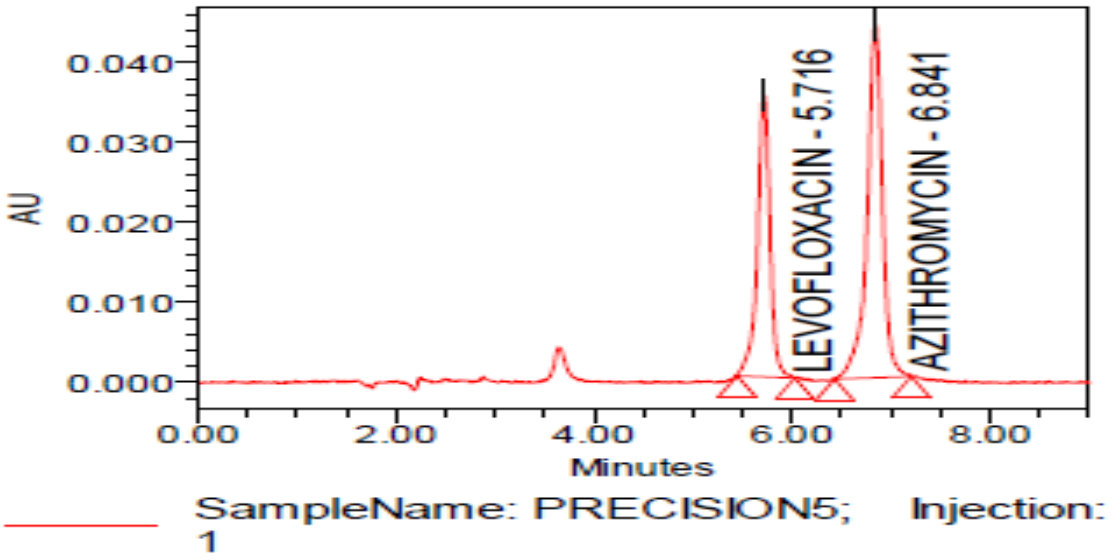


Figure no: 4.3.3.5

Chromatogram showing for precision 6:

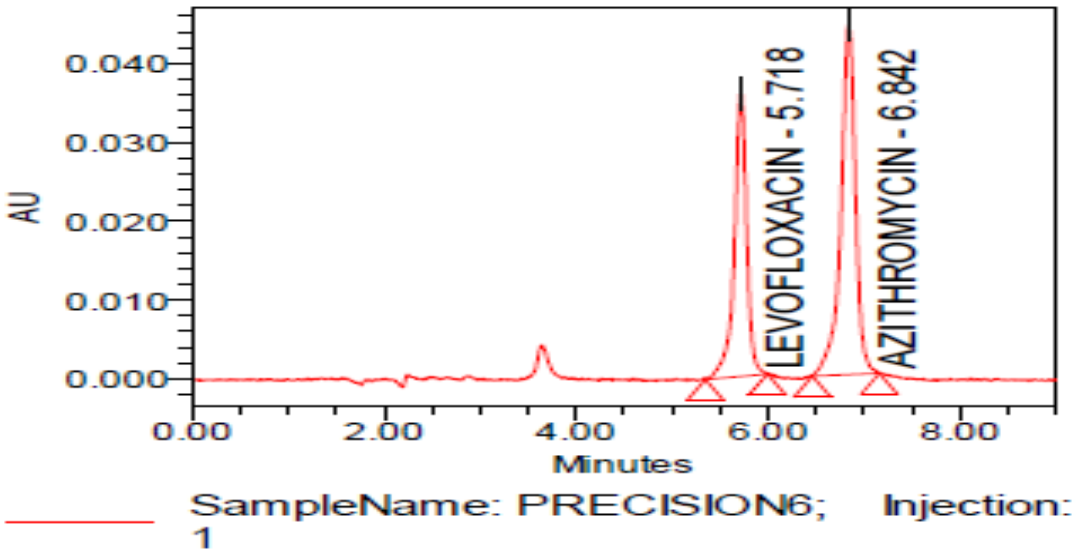


Figure no: 4.3.3.6

4.3.4 LINEARITY &RANGE :

LINEARITY: Linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration within a given range.

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

Preparation of 50% stock solution:

Transfer the 5 mg of Levofloxacin and 5 mg of Azithromycinof working standards into 100 ml volumetric flask and add 60 ml of water and sonicate for 30 mts and dilute to make up with water up to mark.

Preparation of 75% stock solution:

Transfer the 7.5 mg of Levofloxacin and 7.5 mg of Azithromycinof working standards into 100 ml volumetric flask and add 60 ml of water and sonicate for 30 mts and dilute to make up with water up to mark.

Preparation of 100% stock solution:

Transfer the 10 mg of Levofloxacin and 10 mg of Azithromycinof working standards into 100 ml volumetric flask and add 60 ml of water and sonicate for 30 mts and dilute to make up with water up to mark.

Preparation of 125% stock solution:

Transfer the 12.5 mg of Levofloxacin and 12.5 mg of Azithromycinof working standards into 100 ml volumetric flask and add 60 ml of water and sonicate for 30 mts and dilute to make up with water up to mark.

Preparation of 150% stock solution:

Transfer the 15 mg of Levofloxacin and 15 mg of Azithromycinof working standards into 100 ml volumetric flask and add 60 ml of water and sonicate for 30 mts and dilute to make up with water up to mark.

Procedure:

place each level into the chromatographic system and measure the peak area.

Plot a graph of peak area vs concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

The linearity of calibration curves (analyte to peak area ratio V_s concentration) in pure solution was checked over the concentration ranges of 50 - 150 µg/ml for the Levofloxacin and 50-150 µg/ml for Azithromycinrespectively. The linearity was evaluated by linear regression analysis, using least squares method.

The calibration curves were linear in the studied range and equations of the regression analysis obtained for Levofloxacin and AzithromycinY = 3130.x+1350(r^2 = 0.999), and Y = 4697.x+2014 (r^2 = 0.999) respectively.The mean ± standard deviation (SD) for the slope, and correlation coefficient of standard curves were calculated.

The slope and Correlation coefficient values for Levofloxacin were found to be 3130 and 0.999 respectively.

The slope and Correlation coefficient values for Azithromycinwere found to be 4697 and 0.999 respectively.

Table no: 4.3.4.1Linearity data for Levofloxacin and Azithromycin:

Concentration of Levofloxacin (%)	**Peak Area of Levofloxacin (Mv)**	**Concentration of Azithromycin(%)**	**Peak Area of Azithromycin (Mv)**
50%	161184	50%	234242
75%	234183	75%	345290
100%	317107	100%	469082
125%	387932	125%	579692
100%	472914	100%	708226

Linearity of Levofloxacin:

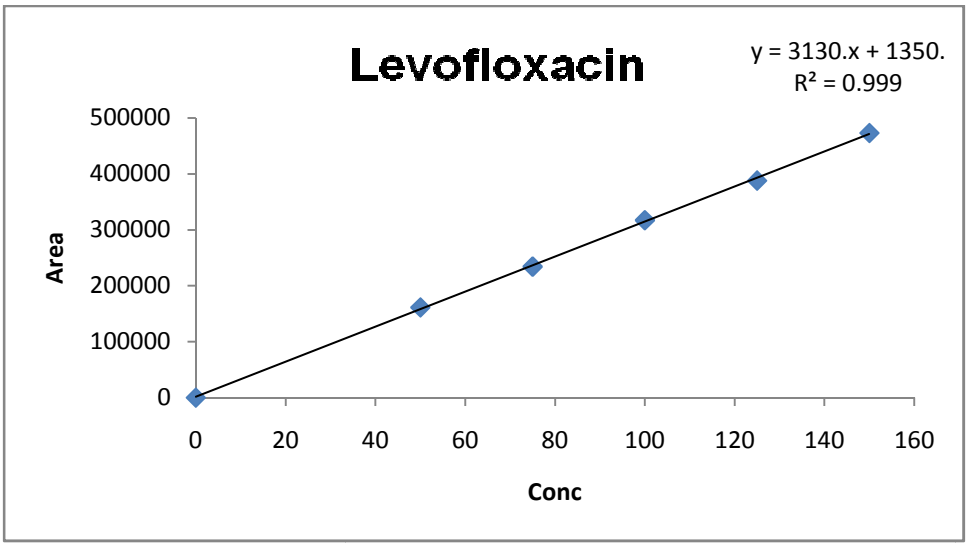


Figure no: 4.3.4.1

Linearity of Azithromycin:

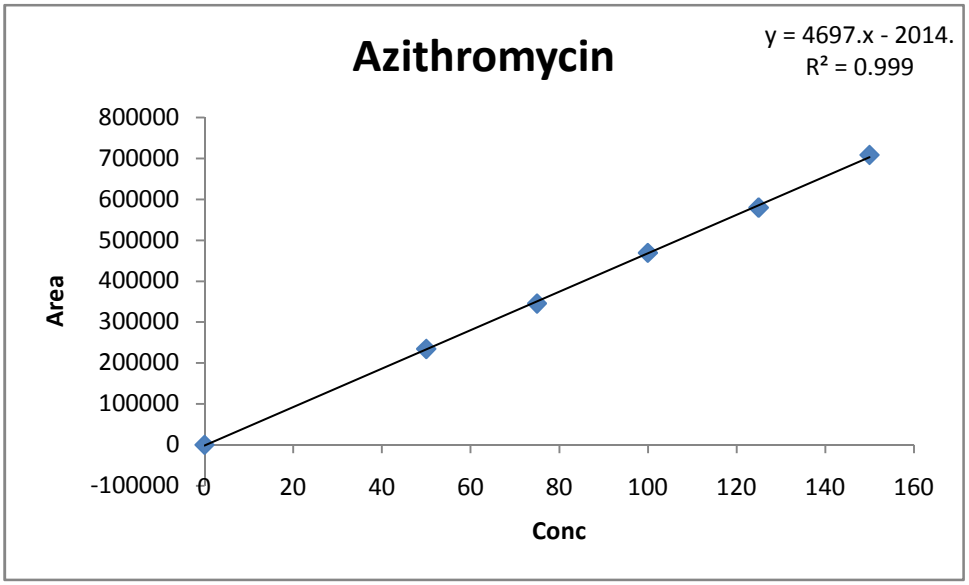


Figure no: 4.3.4.2

Table no. 4.3.4.2Analytical performence parameters for Levofloxacin and Azithromycin:

parameters	Levofloxacin	Azithromycin
Lineariy range	50 – 150%	50 – 150%
Correlation coefficient	0.999	0.999
slope	3130	4697

Acceptance criteria:

Correlation Coefficient (r^2) should be not less than 0.9990.

Chromatogram forLinearity of 50% solution:

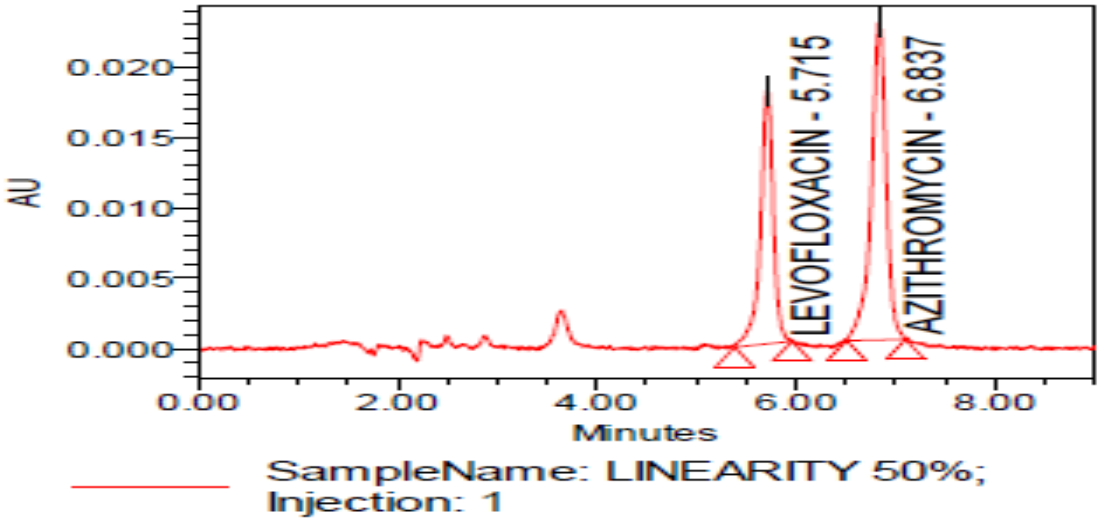


Figure no: 4.3.4.3

Chromatogram for Linearity of 75% solution:

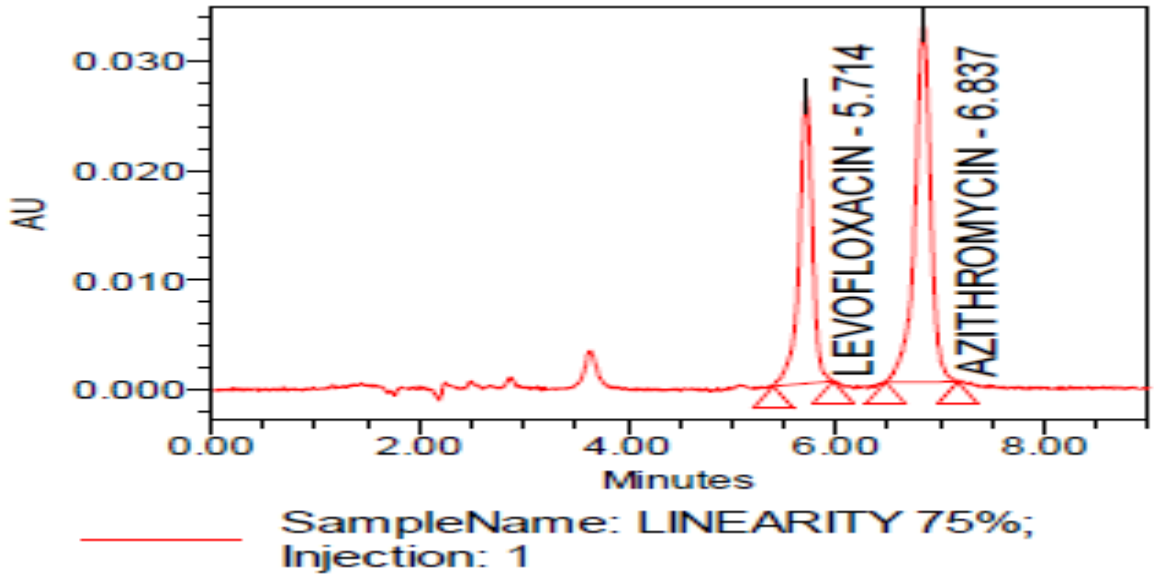


Figure no: 4.3.4.4

Chromatogram forLinearity of 100% solution:

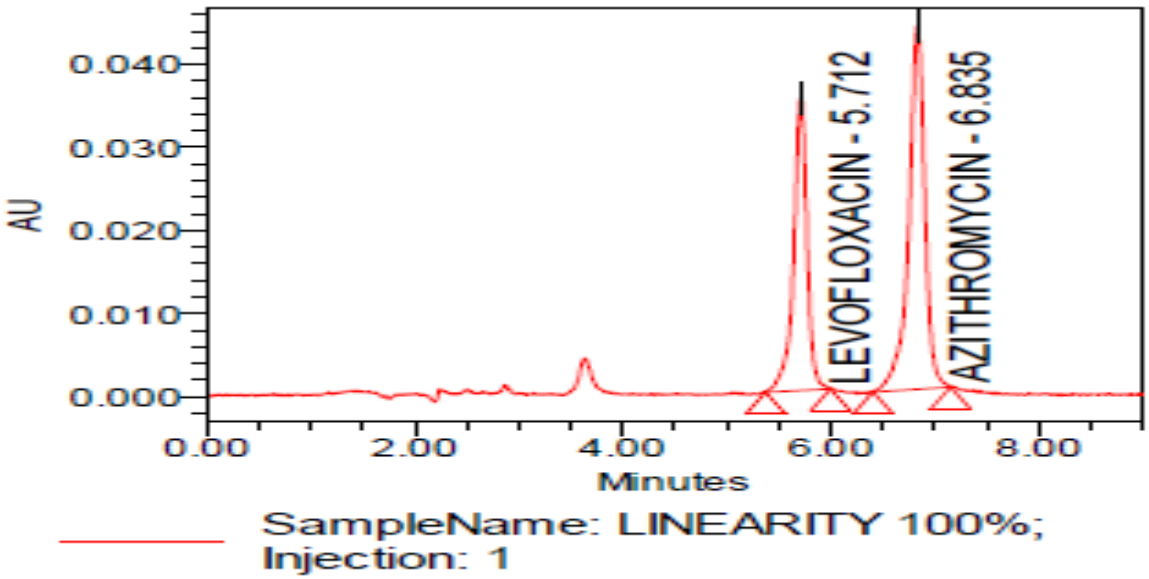


Figure no: 4.3.4.5

Chromatogram forLinearity of 125% solution:

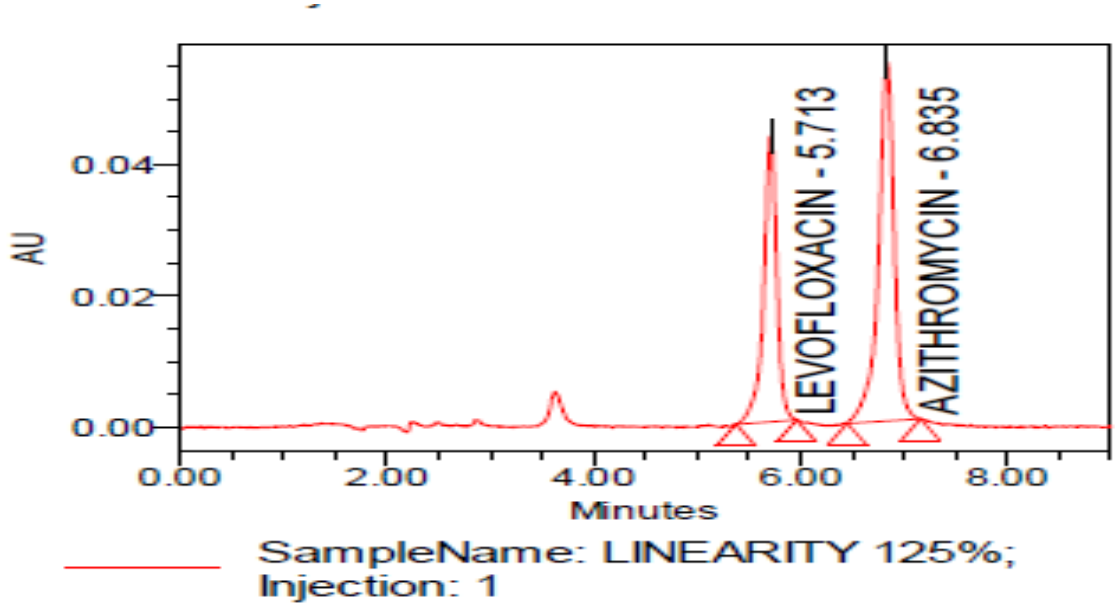


Figure no: 4.3.4.6

Chromatogram forLinearity of 150% solution:

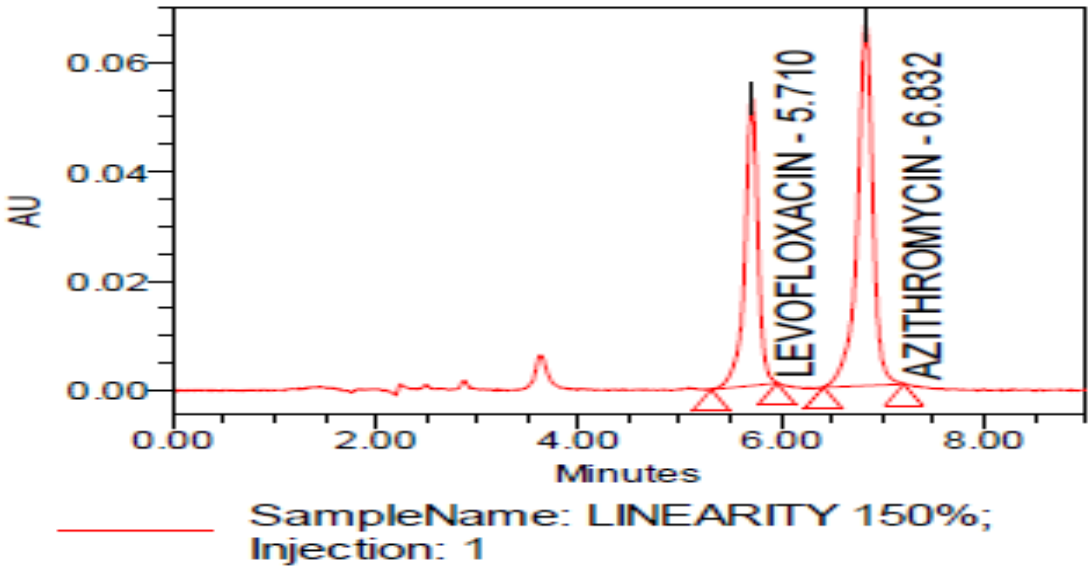


Figure no: 4.3.4.7

Linearity chromatogram for Levofloxacin and Azithromycin 50 – 150 % solution:

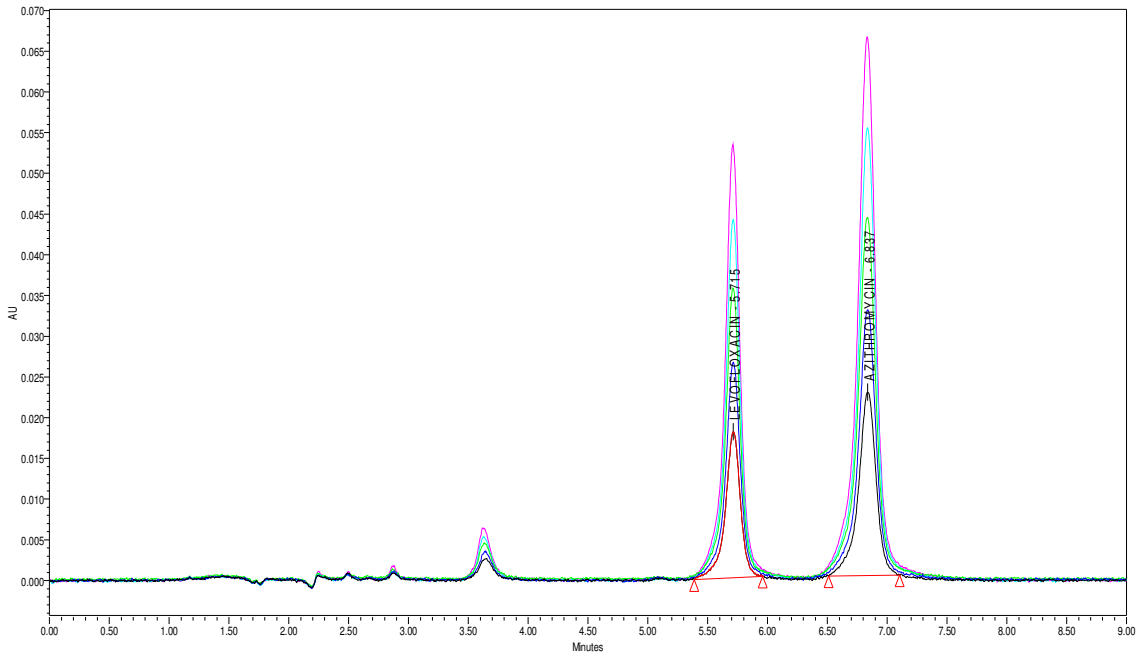


Figure no:4.3.4.8

4.3.5 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.

The specificity study was carried out to check the interference from the excipients used in the formulations by preparing synthetic mixture containing both the drugs and excipients. The chromatogram showed peaks for both the drugs without any interfering peak.

Determination:

Placebo solution was prepared by mixing all the excepients used in the formulation.It was then injected into HPLC system. Blank solution was also injected into HPLC.

Standard solution preparation:

Accurately 10 mg of Levofloxacin and 10 mg of Azithromycinwere weighed and transferred into 100 ml volumetric flask, about 70ml of diluent was added and sonicated for 30 minutes to dissolve it. The volume was made up with diluent. The solution was filtered through 0.45µm membrane filter (Stock solution).

Inject 10µl of standard solution into HPLC system. Compare the chromatograms visually and check for any interference.

Acceptance criteria:

There should not be any peak in the blank & Placebo solution run at the retention time corresponding to Levofloxacin and Azithromycinas in standard run.

Chromatogram showing for specificity – blank solution:

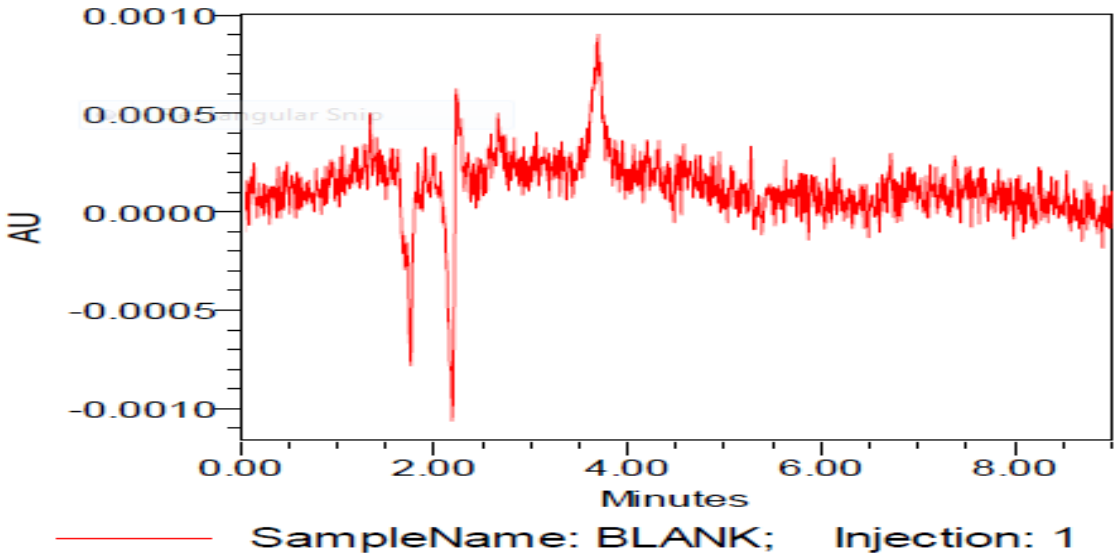


Figure no: 4.3.5.1

Chromatogram showing for specificity – placebo solution:

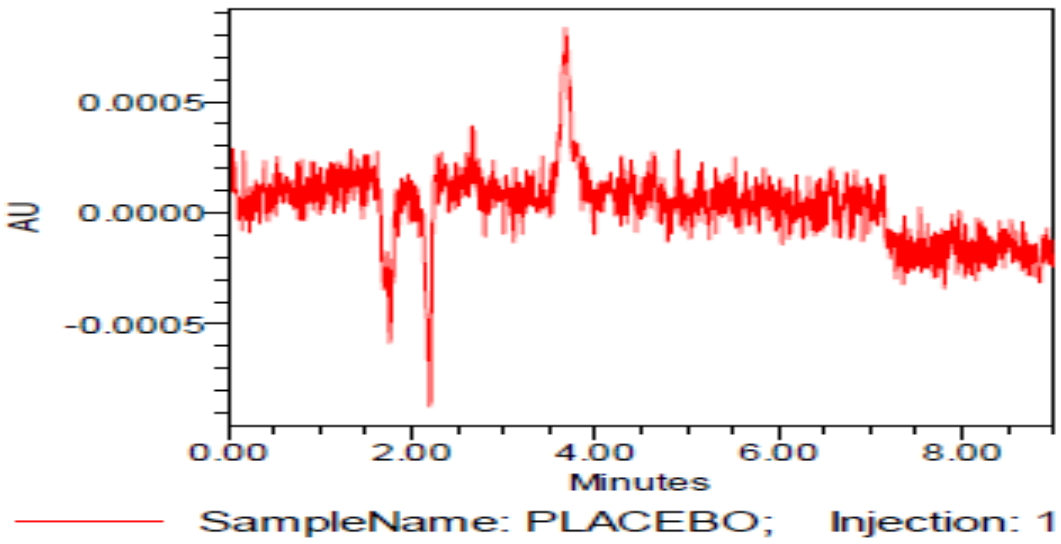


Figure no: 4.3.5.2

Chromatogram showing for the specificity – standard solution :

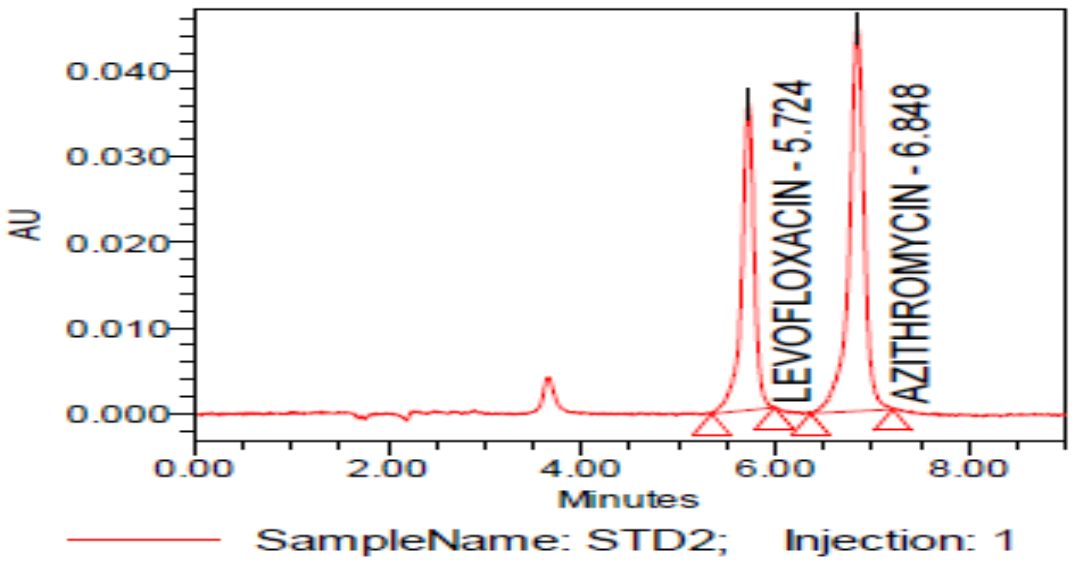


Figure no: 4.3.5.3

4.3.6 ROBUSTNESS :

For demonstrating the robustness of the developed method experimental conditions were purposely altered and evaluated. The method must be robust enough to withstand such slight changes in chromatographic conditions and allow routine analysis of the sample. Effect of column temperature, and flow rate change were carried out and standard was injected. There was no change in system suitability parameters.

Flow rate change - ± 0.2 ml/min

Temperature change - ± 5^0c

Table no. 4.3.6. Robustness studies showing of Levofloxacin and Azithromycin.

Effect	Retention time		Tailing		Plate count	
	LEVO	AZI	LEVO	AZI	LEVO	AZI
Folw rate 0.8µl	6.467	7.580	0.878	0.863	12944	13093
Flow rate 1.2µl	5.120	6.148	0.902	0.970	11426	11882
Temperature 30^0c	8.481	10.214	0.832	0.831	13844	13275
Temperature 40^0c	5.120	6.148	0.832	0.831	11426	11882

Acceptance criteria :

It should pass system suitability parameters

Chromatogram showing for robustness flow 1:

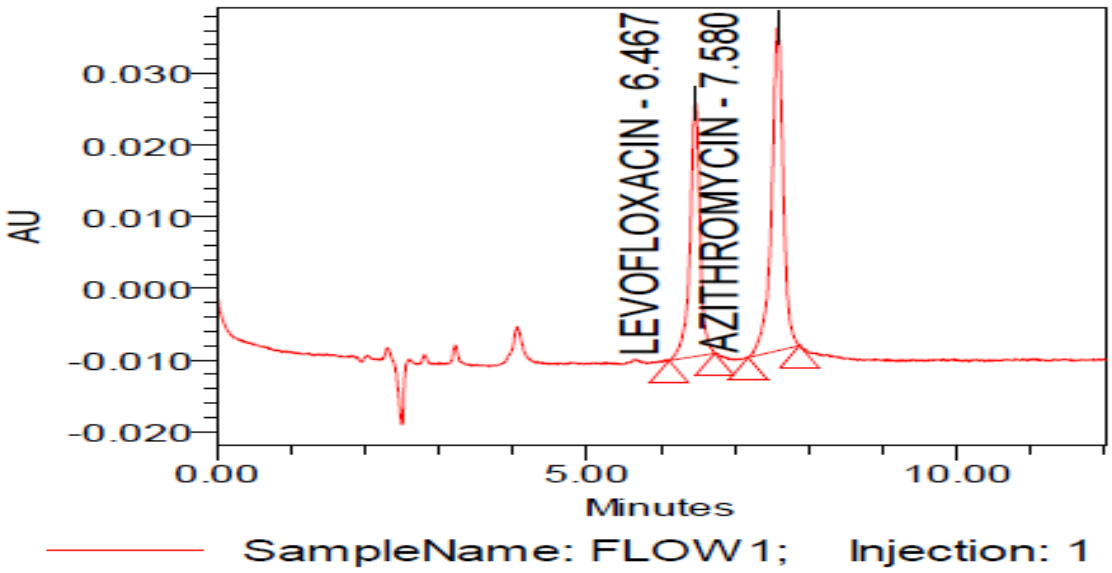


Figure no: 4.3.6.1

Chromatogram showing for robustness flow 2:

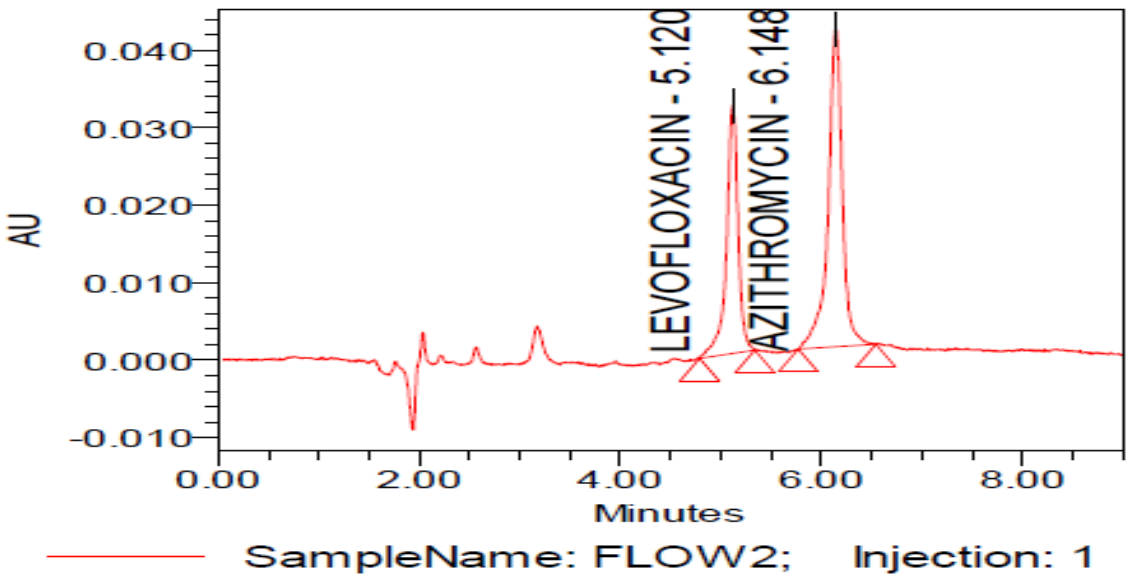


Figure no: 4.3.6.2

Chromatogram showing for robustness temp 1:

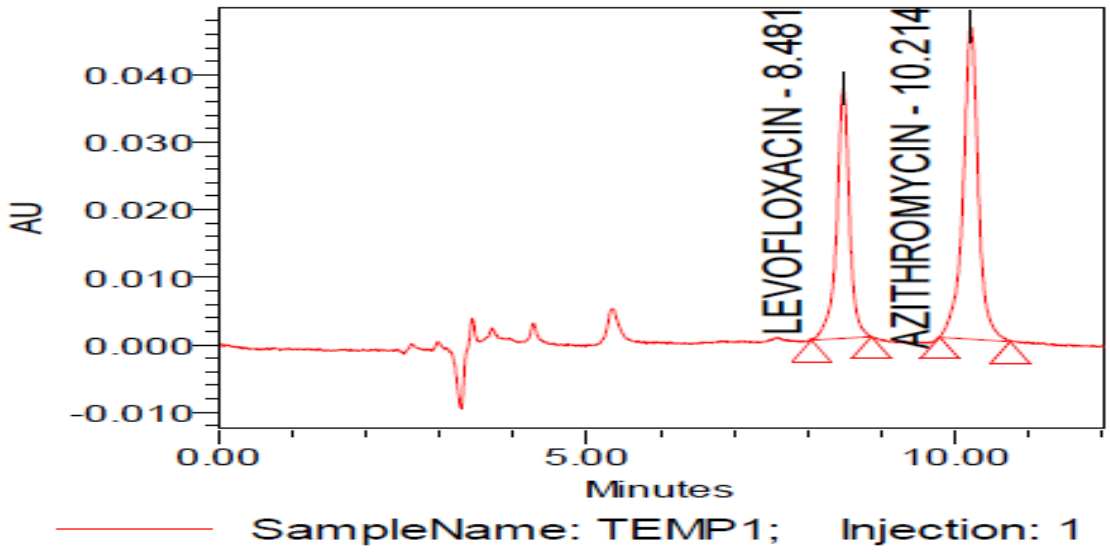


Figure no: 4.3.6.3

Chromatogram showing for robustness temp 2:

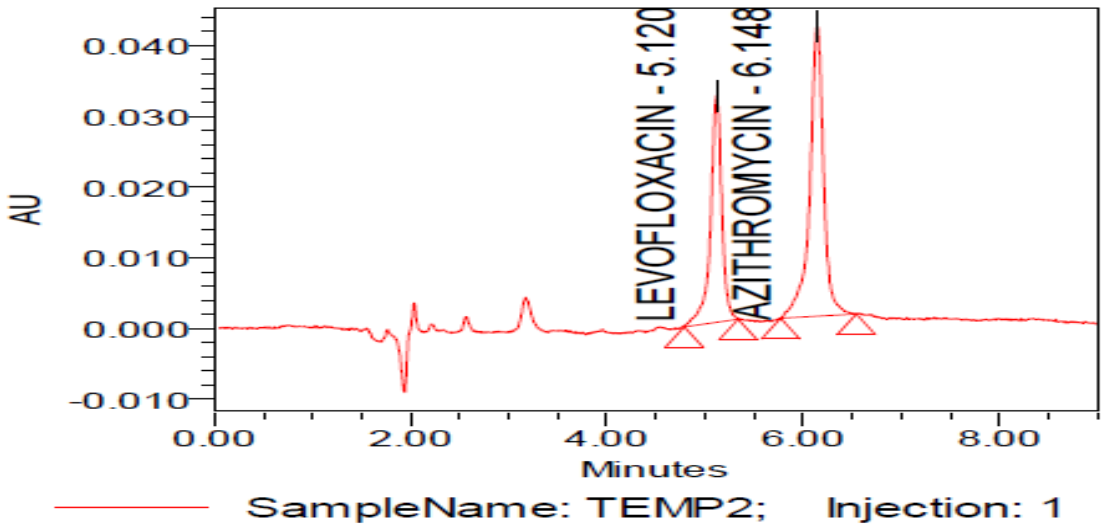


Figure no: 4.3.6.4

4.3.7 LOD& LOQ:

LIMIT OF DETECTION :

Limit of detection is the

lowest concentration of the analyte that can be detected by injecting decreasing amount, not necessarily

quantity by the method, under the stated experimental conditions. The minimum concentration at which the analyte can be detected is determined from the linearity curve by applying the formula.

Sample	LOD	LOQ

LOD = 3/s/n ratio x drug concentration

The lowest concentration of Levofloxacin that can be detected was determined from standard curve was 3.499µg/ml.

The lowest concentration of Azithromycinthat can be detected was determined from standard curve was 2.775µg/ml.

LIMIT OF QUANTIFICATION :

Limit of quantitation is the lowest concentration of the analyte in a sample that can be estimated quantitatively by injecting decreasing amount of drug with acceptable precision and accuracy under the stated experimental conditions of the method .Limit of quantification can be obtained from linearity curve by applying the following formula.

LOQ = 10/s/n ratio x drug conc.

The lowest concentration at which peak can be quantified is called LOQ. It was found to be 11.499µg/ml for Levofloxacin and for Azithromycinwas found to be 9.252µg/ml.

Table no. 4.3.7 LOD & LOQ results showing for Levofloxacin andAzithromycin:

Levofloxacin	**3.449µg/ml**	**2.775 µg/ml**
Azithromycin	**11.499µg/ml**	**9.252µg/ml**

Chromatogram for limit of detection :

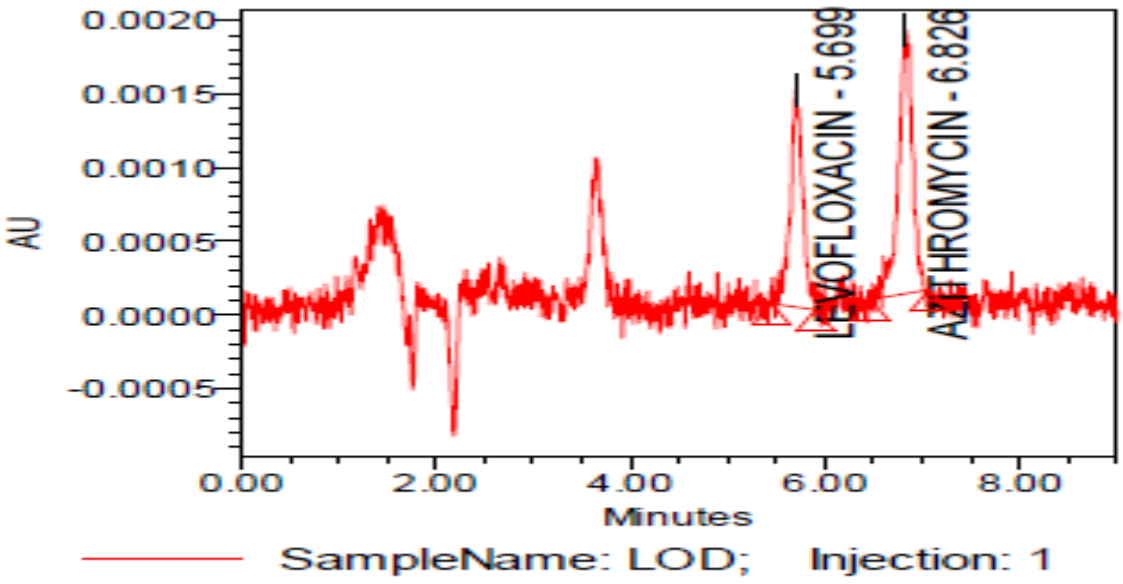


Figure no: 4.3.7.1

Chromatogram for limit of quantification :

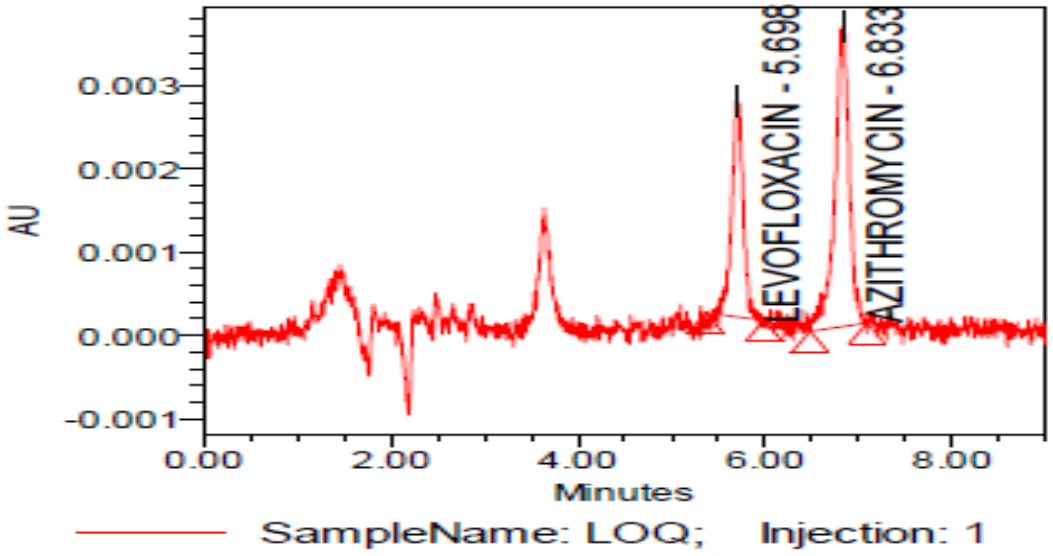


Figure no: 4.3.7.2

4.3.8 DEGRADATION STUDIES :

ACID(0.1N HCL):

Test preparation:

Randomly select 20 tablets from a batch and make it powder, and weigh accurately 2760 mg of powder(equivalent to 1000 mgof Levofloxacin and 1000 mg of Azithromycin) and transfer to 100ml volumetric flask. Then add 10ml of acid(0.1N HCL), shake the flask on a rotator shaker for 30 min and sonicate for 1hr with intermediate shaking then add 10ml base(0.1NaOH) and make up the volume with water.

Pipette out 1 ml of above clear solution and transfer it to 100 ml volumetric flask and make up the volume with water. Inject 10µl of test solution into HPLC system.

BASE(0.1N NaOH):

Test preparation:

Randomly select 20 tablets from a batch and make it powder, and weigh accurately 2760 mg of powder(equivalent to 1000 mg of Levofloxacin and 1000 mg of Azithromycin) and transfer to 100ml volumetric flask. Then add 10ml of base(0.1NaOH) , shake the flask on a rotator shaker for 30 min and sonicate for 1hr with intermediate shaking then add 10ml acid(0.1N HCL), and make up the volume with water.

Pipette out 1 ml of above clear solution and transfer it to 100 ml volumetric flask and make up the volume with water. Inject 10µl of test solution into HPLC system.

PEROXIDE:

Test preparation

Randomly select 20 tablets from a batch and make it powder, and weigh accurately 2760 mg of powder(equivalent to 1000 mg of Levofloxacin and 1000 mg of Azithromycin) and transfer to 100ml volumetric flask. Then add 0.1ml peroxide shake the flask on a rotator shaker for 30 min and sonicate for 1hr with intermediate shaking and make up the volume with water.

Pipette out 1 ml of above clear solution and transfer it to 100 ml volumetric flask and make up the volume with water. Inject 10µl of test solution into HPLC system.

LIGHT:

Test preparation

Randomly select 20 tablets from a batch and make it powder, and weigh accurately 2760 mg of powder(equivalent to 1000 mg of Levofloxacin and 1000 mg Azithromycin) and transfer to 100ml volumetric flask. Then shake the flask on a rotatorshaker for 30 min and sonicate for 1hr with intermediate shaking and make up the volume with water.

Pipette out 1 ml of above clear solution and transfer it to 100 ml volumetric flask and make up the volume with water. Inject 10µl of test solution into HPLC system.

WATER:

Test preparation

Randomly select 20 tablets from a batch and make it powder, and weigh accurately 2760 mg of powder(equivalent to 1000 mg Levofloxacin and 1000 mg of Azithromycin) and transfer to 100ml volumetric flask. Then shake the flask on a rotator shaker for 30 min and sonicate for 1hr with intermediate shaking and make up the volume with water.

Pipette out 1 ml of above clear solution and transfer it to 100 ml volumetric flask and make up the volume with water. Inject 10µl of test solution into HPLC system.

Acceptance criteria:

The net Degardation Should be between 1% to 50%.

Assay formula:

AT WS DT average wt of tablet

---------- x ----------x --------- x ----------x working standard.

AS DS WT lable claim

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

Table no. 4.3.8.Degradation studies showing for the Levofloxacin and Azithromycin:

Test	Area of LEVO	Area of AZI	% Asaay of LEVO	% Assay of AZI	% deg of LEVO	% deg of AZI
Acid	**276538**	**449569**	**85**	**92**	**-14**	**-6**
Base	**279241**	**417170**	**86**	**85**	**-13**	**-13**
Peroxide	**273317**	**430256**	**84**	**88**	**-15**	**-10**
Light	**264009**	**399956**	**81**	**81**	**-18**	**-17**

Water	**295093**	**422565**	**90**	**86**	**-8**	**-11**
Avg assay			**86**	**86**	**-13**	**-12**

Chromatogram showing peaks of acid solution of Levofloxacin and Azithromycin :

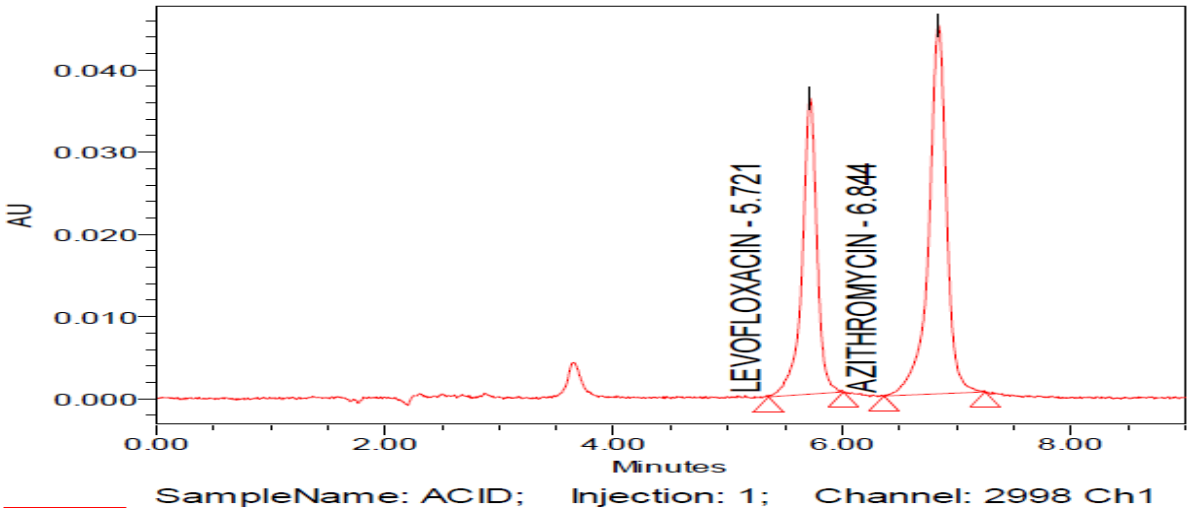


Figure no: 4.3.8.1

Chromatogram showing peaks of base solution of Levofloxacin and Azithromycin :

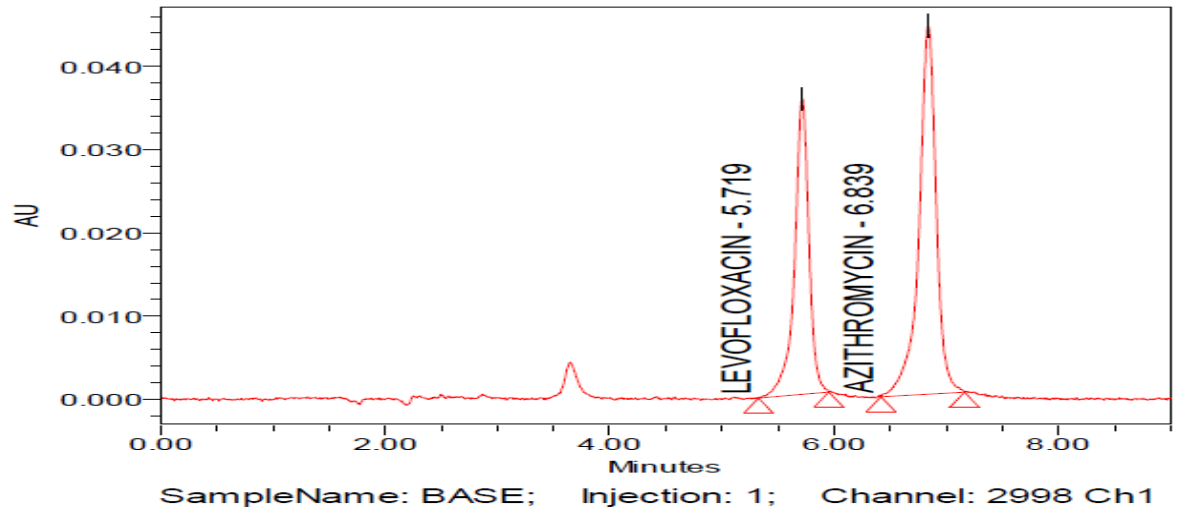


Figure no: 4.3.8.2

Chromatogram showing peaks of peroxide solution of Levofloxacin and Azithromycin :

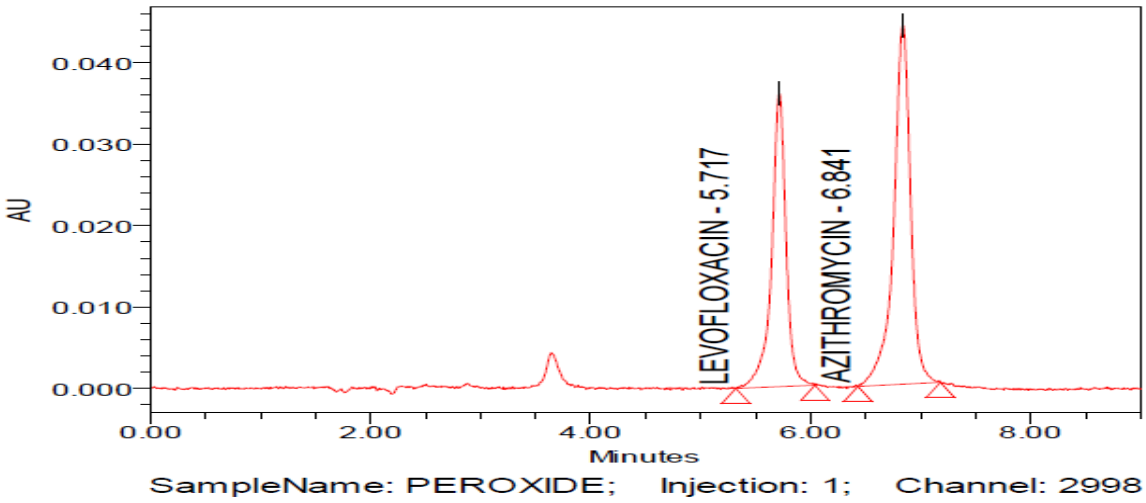


Figure no: 4.3.8.3

Chromatogram showing peaks of light solution of Levofloxacin and Azithromycin :

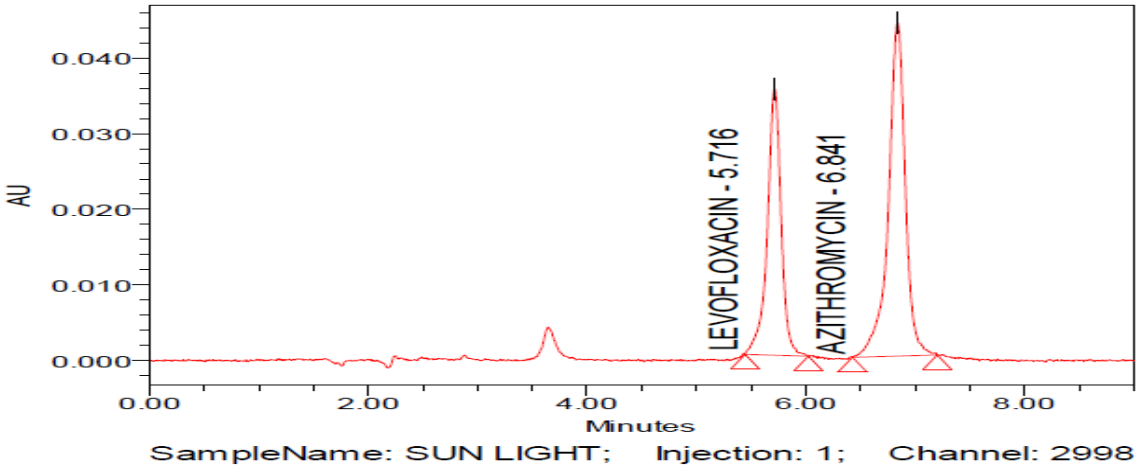


Figure no: 4.3.8.4

Chromatogram showing peaks of water solution of Levofloxacin and Azithromycin:

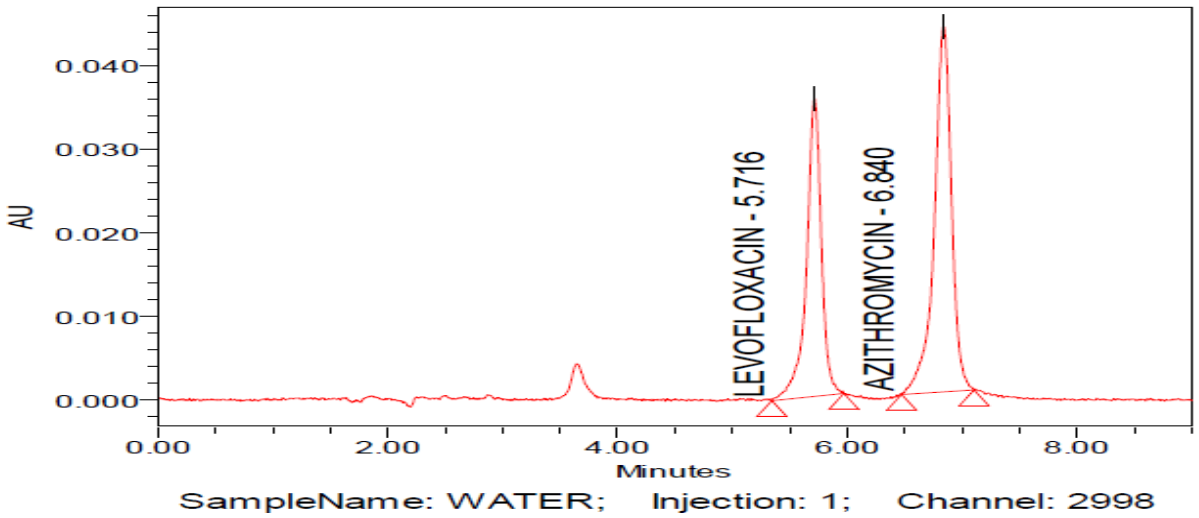
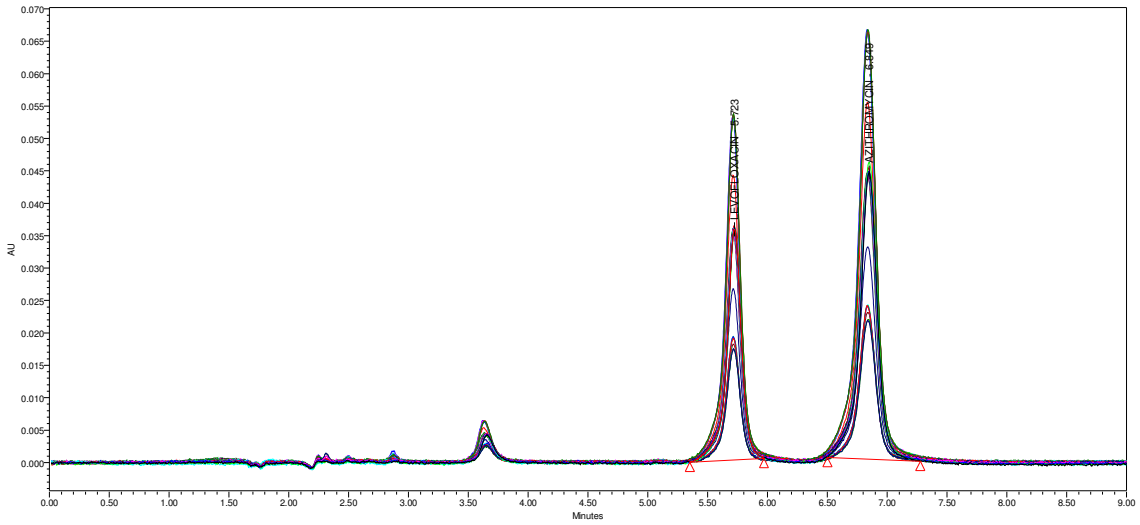


Figure no: 4.3.8.5

Chromatogram showing for the method validation for Levofloxacin and Azithromycin:



5.RESULTS AND DISCUSSION

After several trails with various solvents, mobile phase system composed of KH_2PO_4& K_2HPO_4 buffer and acetonitrilein the proportion of 60:40%v/v. respectively was chosen for the simultaneous estimation of Levofloxacin and Azithromycin in combined dosage form by RP-HPLC. This mobile phase composition offered maximum resolution for the drug at the detection wavelength of 285nm.The column used was C_{18}hypersil (250 × 4.6mm) with flow rate of 1.0 ml/min and UV Detection was carried out.

The individual peaks ofLevofloxacin and Azithromycin were identified by knowing the retention time 5.72 and6.84 minutes respectively.

The results of analysis shows that the amount of drugs was in good agreement with the label claim of the formulation. The tablet shows percentage purity values ranging from 99% of Levofloxacine and 98% of Azithromycinrespectively.

For the System suitability studies were carried out in which the resolution between the peaks, tailing factor and number of theoretical plates was found and these are within the limit.

For the Accuracy of the method was determined by performing recovery studies at 50%, 100%, 150%. The recovery study was carried out and results were expressed in terms of the percentage recovery range found to be within the limit.

For System Precision studies, the standard solution was prepared at working concentration and analysis was carried for five replicated injections. The percentage relative standard deviation (% RSD) was calculated for the peak areas for Levofloxacin and Azithromycin 0.9 and 1.2 and it was not more than 2.0%.

The acceptance criterion of method precision was found to be %RSD NMT 2.0% and the Method Precision for Levofloxacin and Azithromycin shows 1.8 and 1.9and it was not more than 2.0%.

The Linearity for the both drugs**,** From the calibration curve constructed by plotting concentration $v_{s.}$ peak area, it was found that there exists a linear relationship in the concentration range 50 to 150µgm/ml and 50 to 150µgm/ml for Levofloxacin and Azithromycin, with 0.999 and 0.999 as the value of correlation coefficient for the both drugs respectively as These are observed within Limit.

The Robustness of the method was by changing the parameters like flow rate and changing the column temperature and the result was found to be within limit. The method shows no change in the system suitability and precision parameters.

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) Of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method .The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3).The detection limit (LOD) was found to be 3.449µg/ml for Levofloxacin and 2.775µg/mlforAzithromycinrespectively.

The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10).The quantification limit (LOQ) was found to be 11.499µg/ml for Levofloxacin and 9.252µg/ml for Azithromycin respectively.

The percentage degradation of Levofloxacin and Azithromycin Parameters like Acid, Base, Peroxide, Light, and Water studies were found to be within the limits.

The Linearity, precision, accuracy, specificity, repeatability of measurement of peak area as well as repeatability of sample applications are validated as per ICH guidelines and the results are shown in the following **Table.5.1**

S.NO	Parameter	Limits	Observation	
			LEVO	**AZIT**
1	Specificity	No interference	No interference	No interference
2	System precision Method precision	RSD NMT 2.0%	0.8 1.8	1.2 1.9
3	Linearity range	Correlation coefficient NMT – 0.999	0.999	0.999
4	Accuracy	% Recovery range 98 –102 %	100.3	99.6
5	Limit of Detection	Signal noise ratio should be more than 3:1	3.449 µg/ml	2.775 µg/ml
6	Limit of Quantitation	Signal noise ratio should be more than 10:1	11.499 µg/ml	9.252 µg/ml
7	Number of Theoretical Plates	NLT 2000	11519	11695
8	Robustness Change in column temperature, Change inflow		No effect on system suitability parameters	No effect on system suitability parameters
9	Forced degradation	1 - 50%	pass	pass

6.CONCLUSION

The results obtained by the proposed method for determination of Levofloxacin and Azithromycin by RP-HPLC mehod are reliable, accurate, and precise. The values of standard deviation were found satisfactory and the recovery studies were close to 100%. The method does not require prior separation of one drug from other. System suitable parameters, precision, linearity, accuracy, precision, Robustness and stability according to ICH guidelines were found to be satisfactory. The proposed method was simple, specific, requires short time to analyse samples and easy to perform.

Future :

- Hence it was concluded that the RP-HPLC method developed was very much suitable for routine analysis ofLevofloxacin and Azithromycintablet formulations and future planning's use this method for estimation ofLevofloxacin and Azithromycin in clinical trials and Bio analytical studies.

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