ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF OFLOXACIN AND ORNIDAZOLE BY RP-HPLC IN TABLET DOSAGE FORM

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Submitted by

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Under the Guidance of

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CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF OFLOXACIN AND ORNIDAZOLE BY RP-HPLC IN TABLET DOSAGE FORM'** Submitted to The Tamil Nadu Dr. M.G.R.Medical University, Chennai, was carried out G.LAVANYA (Reg: No.261230702),for the partial fulfillment for the degree of MASTER OF PHARMACY in pharmaceutical analysis under the guidance of Mr. R. VIJAY AMIRTHARAJ M.Pharm., HOD,Department of pharmaceutical Analysis J.K.K.Munirajah Medical Research Foundation College of pharmacy, Komarapalayam, during the academic year 2013-2014.

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DECLARATION

The work presented in this dissertation entitled **"ANALYTICAL METHOD DEVELOPMENT AND METHOD VALIDATION OF OFLOXACIN AND ORNIDAZOLE BY RP-HPLC IN TABLET DOSAGE FORM"** was carried out by me, under the guidance of **Mr. R. VIJAY AMIRTHARAJ M.Pharm.,** HOD, Department of pharmaceutical Analysis J.K.K.Munirajah Medical Research Foundation College of pharmacy, Komarapalayam.

This work is original and has not been submitted in part or full for the award of any other degree or diploma of any other university.

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LIST OF ABBRAVATIONS

API	Active pharmaceutical Ingredient
CGLP	Current Good Laboratories Practices
CGMP	Current Good Manufactures Practices
CV	Co-efficient of Variation
°c	Degree centigrade
FTIR	Fourier Transmission Infra Red
ICH	International Conference on Harmonization
K	Capacity factor
LOD	Limit of detection
LOQ	Limit of Quantitation
mg	Milligram
ml	Milliliter
mg/ml	Milligram per milliliter
N	Plate number
NLT	Not less than
NMT	Not more than
nm	Nanometer
PDA	Photo Diode Array
RI	Refractive Index
RP-HPLC	Reverse phase High Performance Liquid Chromatography
RS	Resolution
RSD	Relative Standard Deviation
RT	Retention time
SD	Standard Deviation
T	Tailing factor

USP	United states pharmacopeia
UV	Ultra violet
v/v	Volume per volume
µg	Micro gram
µg/ml	Microgram per Milliliter

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INTRODUCTION

Pharmaceutical analysis deals not only with medicaments (drugs and formulations), but also with their precursors i.e. with the raw material whose degree of purity, which in turn decides the quality of medicaments. The quality of a drug is determined, after establishing its authenticity, which is carried by testing its purity and the quality of the pure substance in the drug and its formulations.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC):

High-performance liquid chromatography is a chromatographic technique used to separate the components in a mixture, to identify each component, and to quantify each component .The method involves a liquid sample being passed over a solid adsorbent material packed into a column using a flow of liquid solvent. Each analyte in the sample interacts slightly differently with the adsorbent material, thus retarding the flow of the analytes. If the interaction is weak, and the analytes flow off the column in a short amount of time, and if the interaction is strong, then the elution time is long.

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases**.** (Sharma B.K.1994)

The HPLC method was considered the choice of estimation, since this method is the most powerful of all chromatographic and other separative methods. The HPLC method has enabled analytical chemist to attain great success in solving his analytical problems. The HPLC is the method of choice in the field of analytical chemistry,

since this method is specific, robust, linear, precise, and accurate and the limit of detection is low and also it offers the following advantages.

The schematic representation of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components .A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or Refractive index (RI).

SCHEMATIC REPRESENTATION OF HPLC SYSTEM

COMPONENTS OF HPLC SYSTEM:

Pump

Pump generates a flow of elute from the solvent reservoir to the system. Most pumps used in current LC system generate the flow by back-and forth motion of a motor –driven piston. (Reciprocating pumps). Because of this piston motion, it produces "pulses". There have been large system improvements to reduce this pulsation and the recent pumps create much less pulse compared to the older ones. Recent analysis requires very high sensitivity to quantify a small amount of analytes, and thus even a minor change in the flow rate can influence the analysis. Therefore, the pumps required for the high sensitivity analysis needs to be highly precise.

Injector

An injector is placed next to the pump. The simplest method is to use a syringe, and the sample is introduced to the flow of eluent. Since the precision of LC measurement is largely affected by the reproducibility of sample injection, the design of injector is an important factor. The most widely used injection method is based on sampling loops. The use of auto sampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Column

The separation is performed inside the column; therefore, it can be said that the column is the heart of an LC system. The packing material generally used is silica or polymer gels. The eluent used for LC varies from acidic to basic solvents. Most column housing is made of stainless steel, since stainless is tolerant towards a large variety of solvents. However, for the analysis of some analytes such as biomolecules and ionic compounds, contact with metals is not desired, thus polyether ether ketone (PEEK) column housing is used instead.

Detector

Separation of analytes is performed inside the column, Whereas a detector is used to observe the obtained separation .The composition of the eluent is consistent when no analyte is present .While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences. This difference is monitored as a form of electronic signal.

On-line detectors

- Refractive index
- UV/Vis Fixed wave length
- UV/Vis variable wave length
- UV/Vis Diode array
- Fluorescence
- Conductivity
- Mass –Spectrometric (LC/MS)
- Evaporative light scattering

Off-line detector

- FTIR spiral disk monitor requires sample transfer on the germanium disk and following scanning in FTIR instrument.

Recorder

The change in eluent detected by a detector is in the form of electronic signal, and thus it is still not visible to our eyes. Nowadays, computer based data processor (integrator) is more common .There are software that are specifically designed for LC system. It provides not only data acquisition, but features like peak-fitting, base line correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as a noise and causes unstable baseline. Generally used method includes sparging (bubbling of inert gas), use of aspirator, distillation system, and/or heating and stirring. However, the method is not convenient and also when the solvent is left for a certain time period (e.g., during the long analysis), gas will dissolve back gradually. Degasser uses special polymers membrane tubing to remove gases. The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go though the pore. By placing this tubing under low pressure container, it created pressure differences inside and outside the tubing (higher inside the tubing). This difference let the dissolved gas to move through the pores and remove the gas. Compared to classical batch type degassing, the degasser can be used on-line; it is more convenient and efficient.

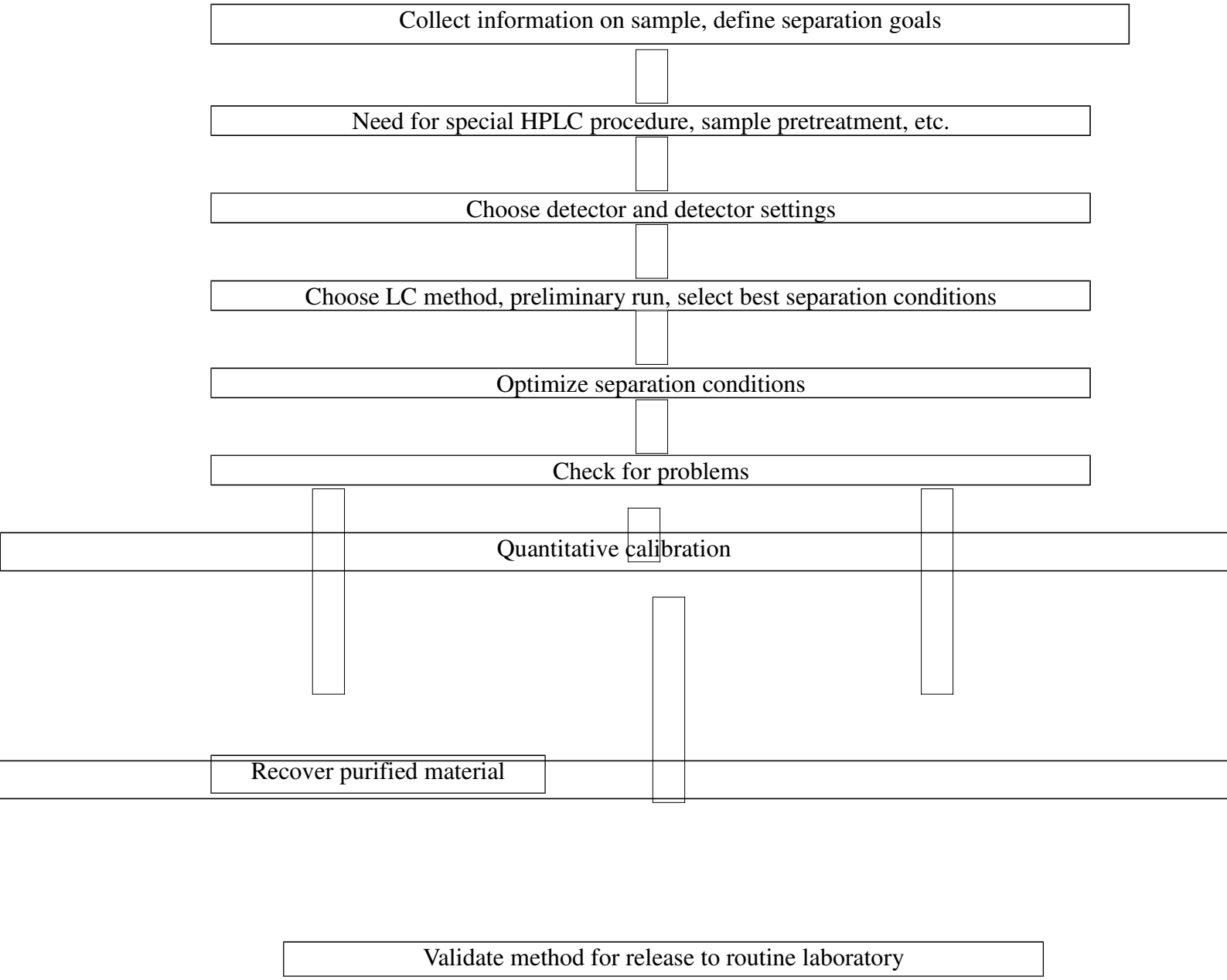
Column heater

The LC separation is often largely influenced by the column temperature. Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperature (50~80^{o}C). It is also important to keep stable temperature to obtained repeatable results even it is analyzed at around room temperature. There are possibilities that small different of temperature causes different separation results. The columns are generally kept inside the column oven (column heater).

(Willard et.al, 1988).

INTRODUCTION TO HPLC METHOD DEVELOPMENT

Method development has following steps:



A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are:

1. Careful sampling and sample preparation.
2. Appropriate choice of the column.
3. Choice of the operating conditions to obtain the adequate resolution of the mixture.
4. Reliable performance of the recording and data handling systems.
5. Suitable integration/peak height measurement technique.
6. The mode of calculation best suited for the purpose.
7. Validation of the development method.

(Synder et.al 1983).

Careful sampling and sample preparation

Before beginning method development, it is need to review what is known about the sample in order to define the goals of separation. The sample related information that is important is summarized in below.

Number of compounds present
Chemical structure
Molecular weight of compounds
pk_a Values of compounds
UV spectra of compounds
Concentration range of compounds in samples of interest
Sample solubility

The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation.

Separation Goals

The goals of HPLC separation need to be specified clearly, which include:

- The use of HPLC to isolate purified sample components for spectral identification or quantitative analysis.
- It may be necessary to separate all degradants or impurities from a product for reliable content assay.
- In quantitative analysis, the required levels of accuracy and precision should be known (a precision of $\pm$ 1 to 2% is usually achievable).
- Whether a signal HPLC procedure is sufficient for a raw material or one or more different procedure are desired for formulations.
- When the number of samples for analysis at one time is greater than 10, a run time less than 20 minutes often will be important.

Sample preparation

Sample come in various forms:

- Solution ready for injection.
- Solutions that require dilution, buffering, addition of and internal standard or other volumetric manipulation.
- Solids must be dissolved or extracted.
- Samples that require pretreatment to remove interference and /or protect the column or equipment from damage.

Most samples for HPLC analysis require weighing and /or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvents is close to that of the mobile phase since this minimizes baseline upset and other problems. Some samples require a partial separation (pretreatment) prior to HPLC, because of need to remove interference, concentrate sample analyte or eliminate "column killers".

The samples may be of two types, regular or special. The regular samples are typical mixtures of small molecules (<2000Da) that can be separated by normal starting conditions. Whereas special samples are better separated under customized conditions given below.

Sample	Requirements
Inorganic ions	Detection is primary problem; use ion chromatography.
Isomers	Some isomers can be separated by reserved-phase HPLC and are then classified as regular samples; better separations of isomers are obtained using
Enantiomers	either (1)normal – phase HPLC or(2) reversed – phase separations with cyclodextrin-silica columns. These compounds require "chiral" conditions for their separation.
Biological	Several factors make samples of this kind "special", molecular conformation, polar functionality, and a wide range of hydrophobicity.
Macromolecules	"Big" molecules require column packing with large pores (>>10-nm diameters); in addition, biological molecules require special conditions as noted above.

Choice of the column

The separation of the column in HPLC is somewhat similar to the selection of columns in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interactions and hydrogen bond formation. In case of ion-exchange chromatography, the separation is based on the differences in the charge, size of the ions generated by the sample

molecules and the nature of ionisable group on the stationary phase. In case of size – exclusion chromatography the selection of the column is based on the molecular weight and size of the sample components. Selection of columns based on the method is briefly summarized in below.

Method /Description /Columns	Preferred Method
Reversed – Phase HPLC Uses water- organic mobile phase Columns: C_{18} (ODS), C8, Phenyl, trimethylsilyl (TMS), and cyano.	First choice for most samples, especially neutral or non-ionized compounds that dissolve in water-organic mixtures
Iron –pair HPLC Uses water – organic mobile phase, a buffer to control pH, and an ion –pair reagent Columns: C_{18} , C_8, Cyano	Acceptable choice for ionic or ionisable compounds, especially bases or cations.
Normal-phase HPLC Uses mixtures of organic solvents as mobile phase. Columns: cyano, diol, amino, silica	Good second choice when reserved-phase or ion-pair HPLC is ineffective; first choice for lipophilic samples that do not dissolve well in water-organic mixtures; first choice for mixtures of isomers and for preparative HPLC

Operating conditions to obtain the adequate resolution of the mixture

Most of the drug come under the category of regular samples mean typical mixtures of small molecules (<2000Da) that can be separated using more or less standardized starting conditions. Regular samples 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. For basic or cationic samples, less acidic reserve phase columns are recommended. Based on recommendations of the conditions, the first exploratory run is carried and then improved systematically. On the basis of the initial exploratory run isocratic or gradient elution can be selected as most suitable. If typical reverse-phase conditions provided inadequate sample retention, it suggests the use of either ion-pair or normal Phase HPLC. Alternatively, the sample may be strongly retained with 100% ACN as mobile phase suggesting the use of non-aqueous reverse phase chromatography or normal phase HPLC.

Method Development

One approach is to use an isocratic mobile phase of some average organic solvent strength (50%). A better alternative is to use a very strong mobile phase first (80 -100%) then reduce % B as necessary. The initial separation with 100% B results in rapid elution of the entire sample, but few groups will separate. Decreasing the solvent strength shows the rapid separation of all components with a much longer run time, with a broadening of latter bands and reduced retention sensitivity. Goals that are to be achieved in method development are briefly summarized in below.

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	< 5 – 10 min is desirable for routine procedures.
Quantization	≤ 2% for assay; ≤5% for less-demanding analyses. ≤15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise rations.
Solvent consumption	Minimum mobile – phase use per run is desirable.

Separation or resolution is a primary requirement in quantitative HPLC .The resolution (Rs) value should be maximum (Rs > 1.5) favors maximum precision. Resolution usually degrades during the life of the column and can vary from day to day with minor fluctuations in separation conditions. Therefore, values of Rs =2 or greater should be the goal during method development for sample mixtures. Such resolution will favor both improved assay precision and grater method ruggedness.

Some HPLC assays do not require base line separation of the compounds of interest (qualitative analysis).In such case only enough separation of individual components is required to provide characteristic retention times for peak identification.

The time required for a separation (runtime = retention time for base band) should be as short as possible and the total time spent on method development is reasonable (runtimes 5 to 10 minutes are desirable).

Condition for the final HPLC method should be selected so that the operating pressure with a new column does not exceed 170 bar (2500psi) and upper pressure limit below 2000 psi is desirable. There are two reasons for this pressure limit, despite the fact that most HPLC equipment can be operated at much higher pressures. First, during the life of a column, the backpressure may rise by a factor of as much as due to the gradual plugging of the column by particulate matter. Second, at lower pressure (<170 bars) pumps, sample valves and especially auto samplers operate much better, seals last longer, columns tend to plug less and system reliability is significantly improved. For these reasons, a target pressure of less than 50% of the maximum capability of the pump is desirable, when dealing with more challenging samples or if the goals of separation are particularly stringent, a large number of method development runs may be required to achieve acceptable separation.

(Skoog et.al, 2009)

Repeatable separation

As the experimental runs described above are being carried out, it is important to confirm that each chromatogram can be repeated. When we change conditions (mobile phase, column, and temperature) between method development experiments, enough time must elapse for the column to come into with the new mobile phase and temperature. Usually column equilibration is achieved after passage of 10 to 20 volumes of the new mobile phase through the column. However; this should be confirmed by repeating the experiment under the same conditions. When constant retention times are observed in two such back – to- back repeat experiments ($\pm$ 0.5% or better), it can be assumed that the column is equilibrated and the experiments are repeatable.

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 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. This approach reduce the time, energy and cost of all instrumental method development.

The various parameters that include to be optimized during method development are

A. Selection of mode of separation.
B. Selection of stationary phase.
C. Selection of mobile phase.
D. Selection of detector.

Selection of mode of separation

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.

Selection of stationary phase

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
- % of carbon loading
- Pore volume
- Surface area
- Reproducibility and reliability
- End capping

In this case, the column selected had a particle size of 5µm and an internal diameter of 4.6mm.The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilities a wide range of columns like dimethyl silane (C_2), butysilane (C_4), octylsilane (C_8),

Octadecyslane (C_{18}), base deactivated silane (C_{18}), BDS phenyl, Cyanopropyl (CN), nitro, amino etc. silica based columns with different cross linking's in the increasing order of polarity are as follows:

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

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

C_{18} was chosen for this study since it is most retentive one. The sample manipulation becomes easier with this type of column. Generally longer columns provide better separation due to higher the theoretical plate numbers. Columns with 5µm particle size give the best compromise of efficiency.

Peak shape is equally important in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result in,

- Inaccurate plate number and resolution measurement
- Imprecise quantitation
- Degraded and undetected minor bands in the peaks tail
- Poor retention reproducibility

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

A column which gives separation of all the impurities and degradants from each other and from analyte peak and which is rugged for variation in mobile phase shall be selected.

Selection of mobile phase

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from each other and from analyte peak.

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. For a given stationary phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. 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 the 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
- pH of the buffer
- Mobile phase composition

Buffers if any and its 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, and Na2HPO4.
- 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 increasingly proportional to retention times. The strength of the buffer can be increasing, if necessary to achieve the required separations. The solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength. The useful pH range for columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above eight, silica may dissolve.

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 selectively 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 $\pm$ 0.2% of the selected mobile phase composition should be used.

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.
- 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. For the greatest sensitivity ƛmax should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wave lengths give greater selectivity.

Performance calculations

Carrying out system suitability experiment does the performance calculations. System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validations have been completed. The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation SD can be determined during validation.

System suitability might then require that retention times fall within a ± 3 SD range during routine performance of the method.

The USP (2000) defines parameters that can be used to determine system suitability prior to analysis include plate number(n),tailing factor(T),resolution(Rs) and relative standard deviation (RSD) of peak height or peak area for respective injections.

The RSD of peak height or area of five injections of a standard solution is normally accepted as one of the standard criteria .For assay method of a major component, the RSD should typically be less than 1% for these five respective injections.

The plate number and / or tailing factor are used if the run contains only one peak. For chromatographic separations with more than one peak, such as an internal standard assay or an impurity method expected to contain many peaks, some measure of separations such as Rs is recommended. Reproducibility of t_R or k value for a specific compound also defines system performance.

The column performance can be defined in terms of column plate number. As the plate count is more the column is more efficient. (Lyoyd.R et.al 1997)

METHOD VALIDATION

The word "Validation'' means "Assessment'' of validity or action of proving effectiveness.

Definition

ICH defines validation as "establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes.''

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Method need to be validated or revalidated.

- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics
- Whenever the method is changed, and the change is outside the original scope of the method.

Purpose of validation

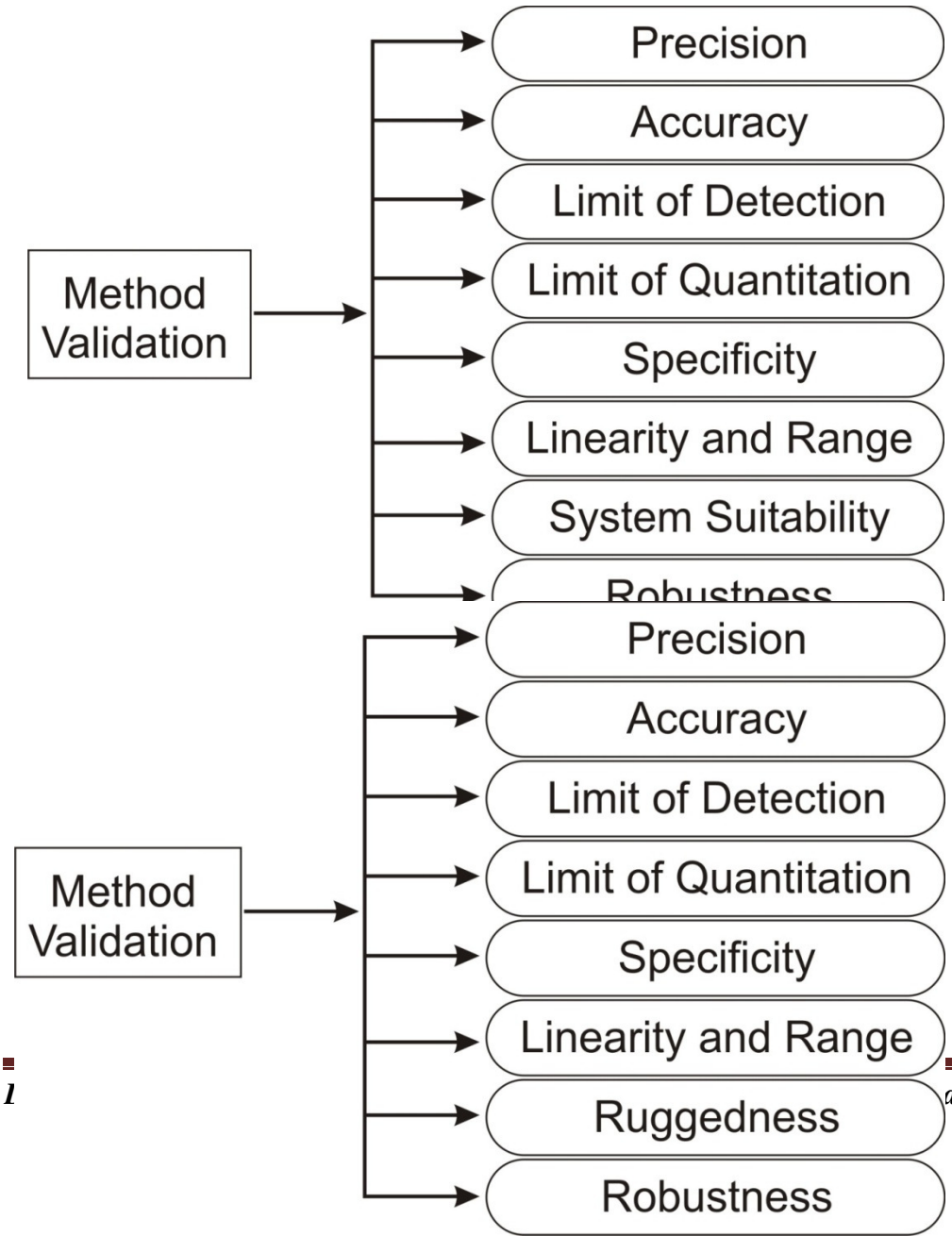
- Enable the scientists to communication scientifically and effectively on technical matter.
- Setting the standards of evaluation procedures for checking compliance and taking remedial action.
- Economic: Reduction in cost associated with process sampling and testing.
- As quality of the product cannot always be assured by routine quality control because of testing of statistically insignificant number of samples.
- Retrospective validation is useful for trend comparison of results compliance to CGMP/CGLP.
- Closure interaction with pharmacopoeial forum to address analytical problems.
- International pharmacopoeial harmonization particularly in respect of impurities determination and their limits.

Method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal

use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do''.

For method validation, these specifications are listed in USP chapter <1225>,and can be referred to as the "eight steps of method validation". as shown in figure below. These terms are referred to as "analytical performance parameters''. or sometimes as "analytical figures of merit.''

In response to this situation, one of the first harmonization projects taken up by the ICH was the development of a guideline on the "Validation of Analytical Methods" Definitions and Terminology. "ICH divided the "Validation characteristics" somewhat differently, as outlined in Figure below



ICH Method Validation Parameters

Method validation parameters

The developed methods were validated by following steps:

A.**Accuracy**

It is defined as closeness of agreement between the actual (true) value and mean analytical value obtained by applying a test method number of times. Spike and recovery studies are performed to measure accuracy; a known sample is added to the excipients and the actual drug value is compared to the value found by the assay. Accuracy is expressed as the bias or the % error between the observed value and the true value (assay value/actual value x 100 %.)

The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. The parameter provides information about the recovery of the drug from sample and effect of matrix, as recoveries are likely to be excessive as well as deficient.

Procedure:

Use a minimum of 3 spiking concentrations in the excipients solution. Prepare two samples of each concentration. Test the 6 samples in triplicate on one run. Measure expected vs. average measured value. Calculate the% recovery.

B.Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample.

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (%RSD) or coefficient of variation (% CV) for a statistically significant number of samples. According to the ICH, precision should be performed at three different levels**:** repeatability, intermediate precision, and reproducibility.

Repeatability is the result of the method operating over a short time interval under the same conditions (or) is the % RSD of multiple determinations of a single sample in a single test run (intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Procedure:

- Prepare three dilution of the sample (high/medium/low concentrations in the range).
- Test 10 replicates of each dilution of the sample.
- Calculate the average and standard deviation for each point on the curve.
- Calculate the RSD for each point on the curve.

Intermediate precision is the results from within lab variations due to random events such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the

individual variables can be monitored (or) intermediate precision (also called inter-assay precision) measure the % RSD for multiple determinations of a single sample, controls and reagents analyzed in several assay runs in the same laboratory.

Procedure:

- Prepare three dilutions of the sample (high/medium/low concentrations in the range).
- Test triplicates of each dilution of the sample inn three different assays.
- Do for day- to –day variations
- Do for lot-to- lot variations of assay materials
- Do for technician – to – technician variation.
- Calculate the average and standard deviation for each point on the curve for each individual test.
- Calculate the RSD for each point on the curve between the assay runs.

Reproducibility refers to the precision between laboratories usually in collaborative studies and not directly relevant to assay validation in a manufacturing facility. Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and the confidence interval.

C.Specificity

It is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. It is not possible to demonstrate that an analytical procedure is specific for a particular analyte. In such case a combination of two or more analytical procedure is recommended to achieve

the necessary level of discrimination. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures or tests.

In case of the assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients and demonstrating that the assay is unaffected by the presence of these extraneous materials. If the degradation product impurity standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g., pharmacopoeia method or other validated analytical procedure (independent procedure). These comparisons should include samples stored under relevant stress conditions (e.g. light, heat humidity, acid/base hydrolysis, oxidation.ect.)

D.Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to- noise ratio, usually two –or three-to-one. The ICH has recognized the signal-to-noise ratio convention, but also lists two other options to determine **LOD:** visual non-instrumental methods and a means of calculating the LOD. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

Procedure

- Prepare a standard concentration of the product in the appropriate solution.
- Prepare a blank solution without any sample (zero concentration).
- Perform the assay at least 3 times in duplicate according to SOP.
- Measure the amount present in the sample and blank.
- Calculate the average for the sample and blank.
- Calculate and standard deviation of the blank.
- Calculate the LOD as 3.3xSD/slope of linearity curve.

E.Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. That is, as the LOQ concentration level decreases, the precision increases. If better precision is required, a higher concentration must be reported for LOQ.

Procedure:

The calculation method is again based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according t the formula: LOQ = 10(SD/S). Again, the standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y- intercepts of regression lines.

F. Linearity:

It is ability of an assay to obtain test results, which are directly proportional to the concentration of an analyte in the sample. The determination of linearity will Identify the range of the analytical assay. It can be measured as slope of the regression line and its variance or as the coefficient of determination (R^2) and correlation coefficient(R).

Procedure:

Determining the coefficient of correlation R for dilutions of the sample over the range claimed for assay.

1. Prepare 6 to 8 sample dilutions across the claimed range
2. Test each dilution in triplicate for 3 runs
3. Record expected values, actual values, and % recoveries for each run
4. Analyze each set of dilutions as a linear curve and calculate R for each assay.

Alternative:

Calculate the accuracy and precisions at each dilution. Range is the highest and lowest concentration with satisfactory accuracy and precision. If the validation study for an analytical test is well planed it should be possible to design the protocol to consider many of the parameters in a single series of test, for instance selectivity (specificity) linearity, range, accuracy and precision for a potency test.

G. Range:

Range is the interval between the upper and the lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. If the relationship between response and concentration is the linear, the range may be estimated by means of a calibration curve.

The range is normally expressed in the same units as the test results obtained by the method. The ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay the minimum specified range is from 80-120% of the target concentration. For an impurity test, the minimum range from the reporting level of each impurity, to 120% of the specification. (For toxic or more potent impurities, the range should be commensurate with the controlled level).

H. Ruggedness:

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lot of reagents, different elapsed assay times, different assay temperatures different days, etc.

I. Robustness:

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. 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. If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variation are:

- Stability of analytical solutions
- Extraction time

In the case of liquid chromatography, examples of typical variations are

- 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.

In the case of gas-chromatography, examples of typical variations are

- Different columns(different lots and/or suppliers)
- Temperature
- Flow rate.

J. System Suitability Test

System suitability test is commonly used to verity resolution, column efficiency and repeatability of the chromatographic system to ensure its adequacy for a particular analysis. According to the United States pharmacopoeia (USP) and the International Conference on Harmonization (ICH), SST is an integral part of many analytical procedure.

Primary SST parameters are most important as they indicate system specificity, precision and column stability. Other parameter include capacity factor (K) and signal to noise ratio(S/N) for impurity peaks.

The USP chromatography general chapter states "System suitability test are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system can be evaluated as such." (USP 36-NF 31, 621 – Chromatography)

INTERPRETASTION OF CHROMATOGRAMS

Figure below represents a typical chromatographic separation of two substances, 1 and 2, where t_1 and t_2 are the respective retention times; and h, h/2, and $W_{h/2}$ are the height, the half – height, and the width at half-height, respectively, for peak1. W1 and W2 are the respective widths of peaks 1 and 2 at the base line. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid

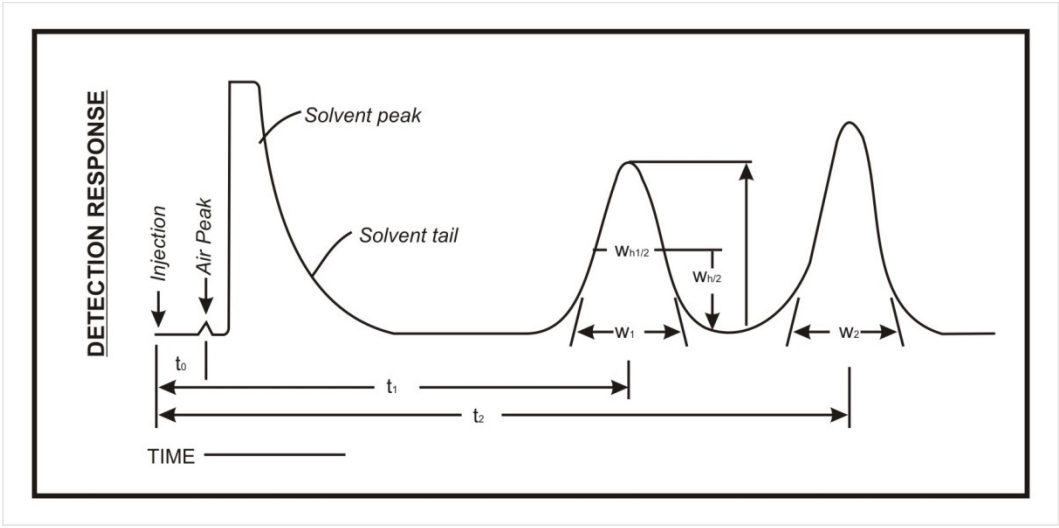


Figure-1

Chromatography retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next.

Relative Retention times:

Relative retention time is calculated by the equation $R_r = t_2/t_1$

t_1 = Retention time of test.

t_2 = Retention time of reference substance, determined under identical experimental conditions on the same column.

Relative Retention:

To calculate the relative retention (r) = $\frac{t_2 - t_M}{t_1 - t_M}$

Where t_M is the retention time of the non-retained marker.

Resolution

The resolution R is a function of column efficiency, N and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug.

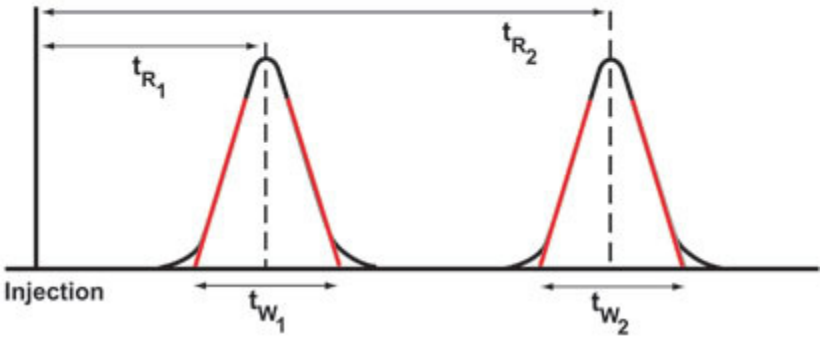


Figure-2

R is determined by the equation:

$$R = \frac{1}{2\left(\frac{t_{2-t_i}}{}\right)} \quad \text{Or}$$

$$R = \frac{2(t_2 - t_2)}{1.70\,(W_{1,\,h/2} + W_{2,\,h/2})}$$

t_2 and t_1 are the retention times of the components.

W_2 and W_1 are the corresponding width at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the base line.

$W_{1h/2}$ and $W_{2h/2}$ are the corresponding peak width at half-height.

Resolution

$$R = \frac{1.18(t_{R2} - t_{R1})}{(W_{h1} + W_{h2})}$$

Where, $t_{R2} > t_{R1}$

t_{R2} and **t_{R1}** = Retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peak

$W_{h1}$and **W_{h2}** = peak width at half height.

Theoretical Plates

Column efficiency also may be specified as system suitability requirements, especially if there is only one park of interest in the chromatograms. The number of the theoretical plates, N, is a measure of column efficiency. It is calculated by the equation.

$$N = 16 [t/w]^2 \text{ or } N = 5.54 [t/w^{1/2}]^2$$

t = Retention time of the substance.

w = width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline.

$W^{1/2}$ = Peak width at half-height.

Precision:

Precision a measure of either degree of reproducibility or of repeatability is determined by making replicate injections of standard preparation and calculating relative standard deviation. Unless otherwise specified in the individual monograph,

data from five replicate injections of the standard preparation are used to calculate the relative standard deviations (SR), if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

Relative Standard Deviation in percentage.

$$SR\ (\%) = \frac{100}{X}\left[\frac{\sqrt{\sum_{i=1}^{i=n}\left(Xi - \bar{x}\right)^2}}{N-1}\right]$$

x = Arithmetic mean of the set.

xi = An individual measurement in a set of N measurements.

N= Number of individuals values

Tailing Factor (or) Symmetry factor

Tailing factor, T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing factor is pronounced (Fig 1). In some cases values less tan unity may be observed. As peak asymmetry increases, integration and hence precision becomes less reliable.

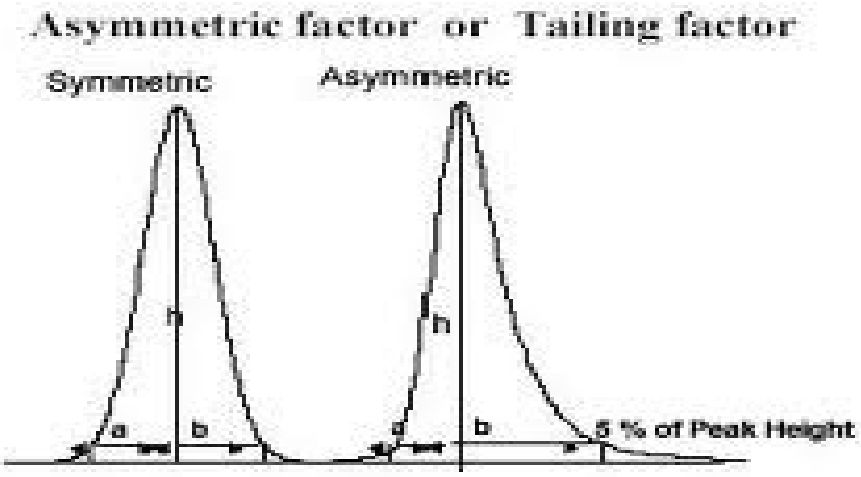


Figure-3

Tailing factor, $T = \frac{W\,0.05}{2F}$

W0.05 = Width of peak at 5% height.

F = Distance from the peak maximum to the leading edge of the peak, the distance is being measured at a point 5% of the peak height from baseline.

Capacity Factor (Mass distribution ratio):

Capacity factor k' of a sample component is a measure of the degree which that component is retained by the column relative to an unretained component

Capacity factor is $k' = \frac{\left(t_{r-t_0}\right)}{t_0}$

t_r – is the elution time of retained component and

t_0 – is the elution time of the unretained sample.

Signal to Noise Ratio:

$$S/N = \frac{2H}{H}$$

Where,

H = Height of the peak corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height.

h = Range of the background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

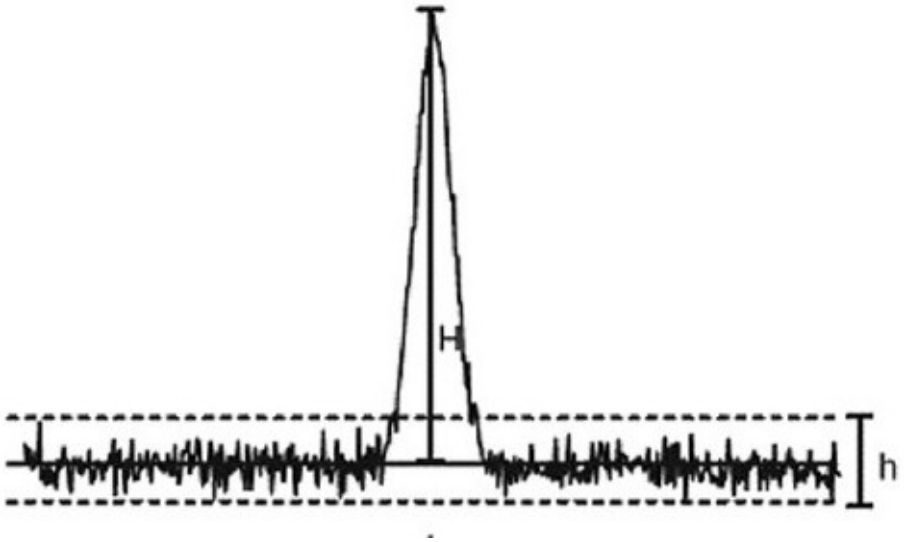


Figure-4

Peak to Valley ratio

The peak-to-valley ratio (p/v) may be employed as a system suitability requirement in a test for related substances when baseline separation between 2 peaks is not reached

$$P/v = \frac{H_p}{H_v}$$

H_p = Height above the extrapolated baseline of the minor peak,

H_v = Height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks.(ICH 2007)

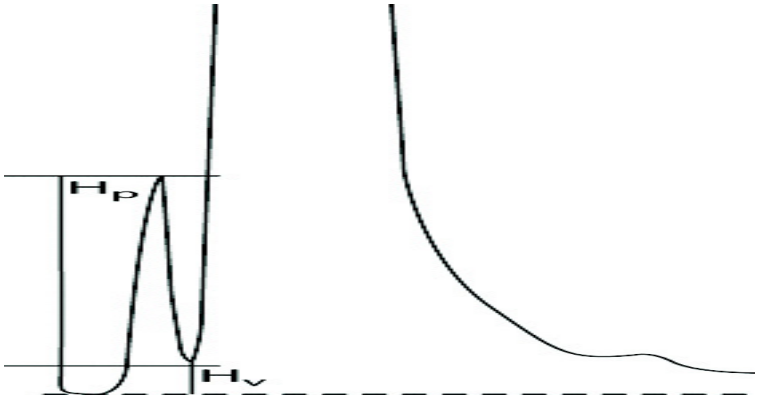


Figure-5

System Suitability Parameters and Recommendations:

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from the other peaks and the void volume, generally k'>2.0
Repeatability	RSD, ≤1% for N≥5 is desirable
Relative retention	Not essential as long as the resolution is stated
Resolution	R_s of >2 between the peak of interest and the closed eluting
Tailing Factor (T)	T of ≤2
Theoretical Plates (N)	In general should be > 2000

STATISTICAL PARAMETERS

Linear regression:

Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the

calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is **y = mx + c**

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$m = \frac{\sum x \sum y - N\sum xy}{(\sum x)^2 - N\sum x^2} \quad \text{and} \quad c = \frac{\sum x \sum xy - \sum y \sum x^2}{(\sum x)^2 - N\sum x^2}$$

Correlation coefficient:

When the changes in one variable are associated or followed by changes in the order it is called correlation. To establish whether there is a liner relationship between two variables x_1 and y_1, use Pearson's correlation coefficient r.

$$r = \frac{n\sum x_1 y_1 - \sum x_1 \sum y_1}{\{n\sum x_1^2 - (\sum x_1)^2\}^{1/2}\{n\sum y_1^2 - (\sum y_1)^2\}^{1/2}}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a define linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1, indicate negative correlation values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

Standard deviation:

The standard deviation measures the spread f the data about the mean value. It is commonly used in statics as a measure of precision statics as a measure of Precision and is more meaningful than is the average and is expressed mathematically

as.

$$x$$
$$Xi-¿$$
$$¿$$
$$¿$$
$$\overline{N-1}$$
$$¿$$
$$¿$$
$$\sum_{i=1}^{i=n} ¿$$
$$S=\sqrt{¿}$$

Where,

S is the standard deviation.

If N is large (50 or more) then of course it is immaterial whether the term in the Denomination is N-1 or N

Σ = sum

X = observed values

$\acute{X}$ = Mean or arithmetic average = ΣX/N

X - $\acute{X}$ = deviation of a value from the mean

N = Number of observations

Percentage relative standard deviation

It is also known as coefficient of variation CV. It is defined as the standard deviation (S.D) expressed as the percentage of mean.

$$\text{CV or \% RSD} = \frac{S.D}{\acute{X}} \times 100$$

Where, S.D = Standard deviation,

$\acute{X}$ = Mean or arthimetic average.

The variance is defined as S^2 and is more important in statistics than S itself.

However, the latter is much more commonly used with chemical data.

Standard Error of mean:

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observation. It is mathematically expressed as

$$\text{S.E} = \frac{S.D}{\sqrt{n}}$$

Where, n = number of observations.
S.D = Standard Deviation(Riley et.al,2009)

Data elements required for essay validation:

It is not always necessary to evaluate every analytical performance parameter, as different test methods require different validation schemes. The most common categories of assays for which validation data should be required are as follows:

i) Quantitation of major components or active ingredients.
ii) Determination of impurities or degradation compounds.
iii) Determination of performance characteristics

Category – I: Analytical methods for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products**.**

Category – II: Analytical methods for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceuticals products. These methods include quantitative essays and limits tests.

Category – III: Analytical methods of determination of performance characteristics (E.g. dissolution, drug release).

The type of method and its intended use dictates which parameters are required to be investigated. They are illustrated in the below.

Data elements required for assay validation

Analytical Performance Parameter	**Assay Category-I**	**Assay category**		**Assay Category III**
		Quantitative	**Limit Test**	
Accuracy	Yes	Yes	*	*
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	*
LOD	No	No	Yes	*
LOQ	No	Yes	No	*
Linearity & range	Yes	Yes	No	*
Ruggedness	Yes	Yes	Yes	*

*may be required, depending on the nature of specific test.

Comparison of Analytical Parameters Required for Assay validation

FDA reviewer Guidance	**USP General Chapter <1225>**	**ICH Q2A Guidelines**
Accuracy	Accuracy	Accuracy
Precision Repeatability Analysis	Precision	Precision
Intermediate precision	No	Intermediate precision
Reproducibility	No	No
Specificity / selectivity	Specificity	Specificity
Detection limit	Detection limit	Detection limit
Quantitation limit	Quantitation limit	Quantitation limit
Linearity	Linearity	Linearity
Range	Range	Range
No	Ruggedness	No
Robustness	Robustness	Robustness
System suitability sample solution stability	System suitability	System suitability

LITERATURE REVIEW

1. Development of UV-spectrophotometric method for the quantititation

Estimation of ofloxacin and ornidazole in cominatined liquid oral dosage form (Ganthi.VM. et.al, 2012.)

The spectrophotometer at medium scanning speed using U.V spectro photometer 119, systronics. The maximum absorbance for ofloxacin and ornidazole were found in 295.6nm and 310.8nm.the linear function of the concentration range of 2 – 10 µg/ml for ofloxacin and 5 - 25µg/ml for ornidazole. The percentage recoveries for ofloxacin and ornidazole were found to be the range of 99.58 - 100.69% and 99.86% 101.0% respectively.

2. Simultaneous spectrophometric estimation of ofloxacin and ornidazole in tablet dosage form. (Bhusari k.P et.al, Jan –Mar 2009.)

The estimation of ofloxacin and ornidazole simultaneously in tablet dosage form. It is shows absorbance maxima at 294 nm and 317nm in N/2 acetic acid, respectively. The linearity was obtained in the concentration range of 2 – 10 µg/ml for ofloxacin and 2 –30µg/ml for ornidazole. The percentage recoveries for ofloxacin and ornidazole were found to be the range of 100.32 %$\pm$ 0.46 and 99.22 $\pm$ 0.59 respectively.

3. A validation RP-HPLC method for simultaneous estimation of cefixime trihydrate and ornidazole in tablet dosage forms. (Sudhakar et.al, Dec 2010.)

The separation and quantification of cefixime trihydrate and ornidazole in tablet dosage form. The determination was carried out using phenomenex C18 column (25 cm x 4.6mm id) as a stationary phase and mobile phase is acetonitrile : 40mM KH2PO4 in proportion of 40:60(v/v) with pH adjusted to 6 + 0.5 by using

triethylamine. The flow rate was 1.0ml/ml and the eluent was monitored at 310nm. The retention time of cefixime trihydrate and ornidazole were 2.75 ± 0.028 min and 6.67 ± 0.018.the coefficient of correlation and percentage recoveries of cefixime trihydrate and ornidazole were 0.9986 and 100.01%, 0.9994 and 99.98% respectively.

4. Spectrometric estimation of cefixime and ofloxacin from tablet dosage form. (Avanija dube et.al, year March 2011.)

The simultaneous estimation of cefixime and ofloxacin from tablet dosage form. Method 1 involves formation of simultaneous equation at 304nm (cefixime) and 296 nm (ofloxacin). Method 2 involves formation of absorbance ratio equation at 275(is absorptive point) using methanol dilution. The linearity was observed in the concentration range of 4- 20 µg/ml for cefixime and 2-10 µg/ml for ofloxacin. Recovery studies are cefixime 97 ± 0.0016 and ofloxacin 102.22 ± 08.

5. Development of colorimetric method for the analysis of Pharmaceutical formulation containing both ofloxacin and cefixime. (Rajnish Kumar et.al, Nov-Dec 2010.)

The determination of ofloxacin and cefixime in same pharmaceutical formulation. ofloxacin forms an orange coloured product in the presence of ferric chloride solution in acidic medium and the absorbance of orange colored species formed was measured at 435nm. linearity concentration range 15 - 75 µg/ml and cefixime forme a product in the presence of fehling solution. greenish coloured Species formed was measured at 490nm against reagent .the linearity concentration is 5to 40µg/ml. Recovery studies is ofloxacin 99.71% and cefixime 99.50%

6. Development and validation for the simultaneous estimation of ofloxacin and tinidazole in tablets. (Dharuman .J et.al, April – June 2009.)

The separation was carried out using a mobile phase consisting of 0.5%v/v triethylamine buffer of pH 3.0 and acetonitrile in the ratio of 73: 27.column used was kromasil C8, 5µ.15 cm x4.6mm id with flow rate of 1.2 ml /min using PDA detection at 303nm. The linear concentration range of 10 – 50 µg/ml and 30 -150 µg/ml for the assay of ofloxacin and tinidazole respectively. The Retention times of ofloxacin, tinidazole were found to be 2.3 and 4.1 min.The limit of LOQ for ofloxacin and tinidazole were found to be 10 and 30µg/ml respectively. Recovery studies is ofloxacin 100.85% $\pm$ 1.256 % and tinidazole 99.58% $\pm$ 0.896.

7. Validated high performance thin layer chromatography method for simultaneous estimation of ofloxacin and ornidazole in tablet dosage form. (Ganthimathi .M et.al, 2006.)

The method employed silica gel 60GF254 TLC precoated plates as stationary phase and a mixture N-butanol: ethanol: ammonia (5:5:4%v/v/v) as mobile phase. The plate was scanned and quantified at 295nm using camag TLC scanner. The RF values of 0.59 $\pm$ 0.02 and 0.86 $\pm$ 0.01 for ofloxacin and ornidazole respectively .The migration distances was 0.82cm .The calculated the peak area was found to be 102.27 $\pm$ 0.15 and 103.04 $\pm$0.21% respectively.

8. Development and validation of column HPLC and derivative spectrophotometric methods for determination of levofloxacin and ornidazole in combined dosage forms. (Patel SA et.al, Jul-Aug 2008.)

The separation was carried out using a mobile phase KH_2PO_4 buffer (pH 6.8) and methanol, acetonitrile (70 + 15 + 15 v/v/v). column used was phenomenox C_{18} 250mm x 4.6mm id, 5µ.with flow rate of 1.5 ml/min using PDA detection at 295nm.The both linear concentration range of 1-10µg/ml.Recovery studies is levofloxacin 101.7% $\pm$ 0.,23 and ornidazole 99.23 % $\pm$ 1.57%.The simultaneous estimation of levofloxacin and ornidazole from tablet dosage

form. It is shows absorbance maximum at 310nm ornidazole and 295nm levofloxacin the linear concentration range of 2-40µg/ml the both levofloxacin and ornidazole. Mean recovery was 99.46% ± 0.96 and 100.9 % ± 0.72.

9. Development and validation of a method for simultaneous estimation of ofloxacin and ornidazole in different dissolution media. (Patel D.M et.al, Jul 2012.)

The estimation of ofloxacin and ornidazole simultaneously in tablet dosage form. It is shows absorbance maximum at 294nm in 0.1M Hcl for ofloxacin and 287nm in phosphate buffer pH 6.8 and phosphate buffer p^H7.4 .Which ornidazole showed absorption maxima at 277nm in 0.1M Hcl and at 319nm in two buffers. The linearity was obtained in the concentration range of 1 – 8 µg/ml for ofloxacin and 4 - 26 µg/ml for ornidazole.

10. Simultaneous estimation of ornidazole and ofloxacin by derivative spectrophotometry method. (Daxina et.al, May 2011.)

In first order derivative spectrophotometry wave length selected for quantitation were 310.9nm for ofloxacin and 297.9nm for ornidazole. In observed in the concentration range of 5 – 25 µg/ml for ornidazole and 2 – 10 µg/ml for ofloxacin. The percentage recoveries for ofloxacin .The percentage recoveries for ofloxacin and ornidazole were found to be the range of 98.06% - 100.14%, and 99.13% - 101.2% respectively.

11. A validated RP –HPLC method for simultaneous estimation of cefixime and ornidazole in tablet dosage forms. (Satish shelke et.al, July 2012.)

The separation and quantification of cefixime and ornidazole in tablet dosage form. the determination was carried out using Kromasil 100 C_8 3.5µm Column (150mm x 4.6mm id) as a stationary phase and mobile phase is water(pH 3.1) : acetonitrile in proportion 75:25 and adjust pH by using orthophosphoric acid. The flow rate was 1.0ml/min and the eluent was monitored at 288nm. The retention time of cefixime and ornidazole were 3.34min and 6.1min respectively.The coefficient of correlation and percentage recoveries of cefixime and ornidazole were 0.9983 and 99.86% and 0.9981 and 100.28% respectively.

12. A validation RP HPLC method for simultaneous estimation of Nitazoxanide and ofloxacin in tablet dosage form. (Sharma .S.et.al, Jan – Feb 2011.)

The estimation of nitazoxanide and ofloxacin in tablet dosage form.The determination was carried out using H1Q SIL C_{18} column (250 x 4.6mm id) as a stationary phase and mobile phase is acetonitrile: methanol: 0.4M citic acid (60: 30: 10 v/v/v) the flow rate was 0.5ml/min and the eluent was monitored at 304nm.The percentage mean recovery of ofloxacin was found to be 99.93% nitazoxanide was found to be 99.86%.

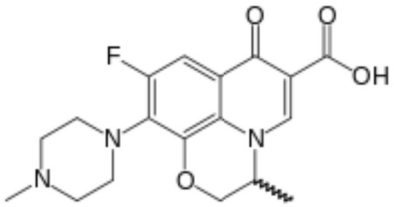
13. Development and validation of a method for simultaneous estimation of ofloxacin and ornidazole in different dissolution medium. (Dasharath M.patel.et.al, Jul- Dec 2012.)

The estimation of ofloxacin and ornidazole simultaneously in tablet dosage form.It shows absorbance maximum at 294nm in 0.1N Hcl for ofloxacin and ornidazole at 277nm.It shows absorbance maximum at 287nm in both phosphate buffer for ofloxacin and 319nm for ornidazole .The linearity observed was in the concentration range of 1 -8 µg/ml and 4 - 25µg/ml for ofloxacin and ornidazole respectively.

DRUG PROFILE

Generic Name : Ofloxacin

Structure :



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

IUPAC name : (±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1- piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine - 6-carboxylic acid.

Molecular weight : 361.368g/mol

Description : A pale yellow or bright yellow, crystalline power

Solubility : Soluble in glacial acetic acid, slightly soluble in water, dichloromethane and methanol.

pKa : 6.22

Category : Anti bacterial activity

Pharamacokinetic data

Bioavailability : 85%-95%

Protein binding	:	32%
Metabolism	:	Metabolized via the liver, extract in the Urine
Half life	:	8-9hours
Excertion	;	kindly(as conjugates and metabolites)

Mechanism of action

Ofloxacin is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria. If function by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, which is an enzme necessary to separate replicated DNA, there by inhibiting cell division .The floroquinolones interface with DNA replication by inhibiting an enzyme called DNA gyrase.

Contra indication:

Ofloxacin is now considered to be contraindicated for the treatment of certain sexually transmitted diseases. There is one contraindication now found within the

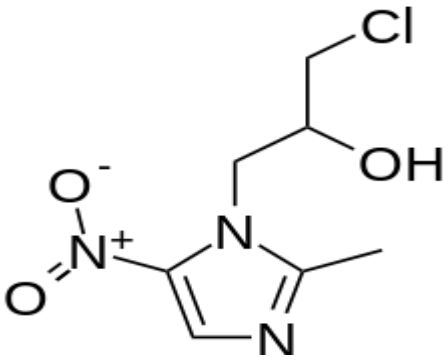
2008 package insert for floxin. That being that Floxin is to be avoided in patients with a known hypersensitive to ofloxacin or other quinolone drugs. Caution in patients with liver function disorders (e.g., cirrhosis with or without acites). Ofloxcian is also considered to be contraindicated within the pediatric population, pregnancy, nursing mothers, patients with psychiatric illnesses and in patient with epilepsy or other seizure disorders.

Interaction:

Interaction increased the risk of cardio toxicity and arrhythmias, anticoagulant effect, the formation of non- absorbable complexes, as well as increasing the risk of toxicity. Concurrent administration of ofloxacin, with magnesium or aluminum antacids, sucralfate or product containing calcium, iron or zine may substainly decrease the absorption of ofloxacin, resulting in serum and urine levels considerably lower then desired.(K.D tripathi 2004)

Generic Name : Ornidazole

Structure :



Molecular Formula : $C_7H_{10}ClN_3O_3$

IUPAC Name : 1-chloro-3-(2-methyl-5-nitro-1*H*-imidazol-1-yl) propan-2-ol **Molecular weight** : 219.625 g/mol

Description : A white to yellowish white crystalline powder

Solubility : Soluble in chloroform and in methanol

Pka : 2.5

Category : Antiprotozoal

Mechanism of action:

Ornidazole is a 5-nitroimidazole derivative active against protozoa and anaerobic bacteria. It is converted to reduction products that interact with DNA to cause destruction of helical DNA structure and strand leading to a protein synthesis inhibition and cell death in susceptible organisms. (www.drug bank .com)

Pharmacodynamics:

Absorption: Readily absorbed (oral and intravaginal); peak plasma concentrations after 2 hr (oral), 12 hr (intravaginal).

Distribution: Body tissues and fluids (wide), CSF. Protein-binding: <15%.

Metabolism: Hepatic.

Excretion: Via urine (as conjugates and metabolites), via faeces (small amounts); 12-14 hr (elimination)

Indication: Treatment of parasitic infections, including amebiasis, giardiasis, and Trichomonas vaginalis; treatment of infections due to susceptible strains of anaerobic bacteria.

Contra indication: Hypersensitivity to ornidazole or to other nitroimidazole derivatives.

Adverse reaction: Somnolence, headache, nausea, vomiting, dizziness, tremor, rigidity, poor coordination, seizures, tiredness, vertigo, temporary loss of consciousness and signs of sensory or mixed peripheral neuropathy, Taste disturbances, abnormal LFTs, skin reactions.

Drug interaction: Potentiates effect of coumarin-type oral anticoagulants. Prolongs the muscle-relaxant effect of vecuronium bromide. See Below for More ornidazole Drug Interactions.

Combined drug uses:

Ofloxacin and ornidazole can be used in the management of a variety of infections caused by bacteria, fungi, and parasites. They are available in a combination pill in some regions for the convenience of patients; the drugs can also be taken independently. A medical provider may recommend this option to a patient who appears to be a good candidate for therapy on the basis of diagnosis and history. It is not advised for people who react badly to quinolone antibiotics or who have known allergies to either drug. One common use of this drug combination is in the management of gastrointestinal infections that cause diarrhea and discomfort, including infections with amoebae and protozoan's. Patients can also take it for urinary tract infections, as well as fungal infections of the oral cavity and vagina. Another application for ofloxacin and ornidazole is treatment of respiratory infections like bronchitis and pneumonia complicated by the presence of aggressive bacteria.

Courses of therapy may be relatively short in all these cases, typically between three and five days. Some sexually transmitted infections, like gonorrhea and Chlamydia, are also treatable with ofloxacin and ornidazole. The short course of therapy can be useful in the management of such conditions as it increases the chances that the patient will adhere to the therapy, eliminating the infectious organisms. Patients who complete their courses of drugs are less likely to experience a recurrence or contribute to the development of drug resistance that could make future infections harder to treat.

A combination of ofloxacin and ornidazole can also be used to treat Hansen's disease, or leprosy. The medications can be used as part of a larger treatment plan that may include other drugs. They can be very effective in a short period of time, reducing the amount of time the patient needs to spend in treatment to eliminate the bacteria responsible for the disease.

In addition to benefiting the patient, this helps prevent the spread of the organisms to the rest of the community. People taking ofloxacin and ornidazole can experience side effects like increased light sensitivity and gastrointestinal discomfort. It is also possible to have severe side effects like rashes and difficulty breathing, which indicate an allergic reaction to one or both medications. If these develop, the patient should discuss them with a medical provider to determine the next step, like switching to a different drug to treat the infection.

AIM AND PLAN WORK

For the simultaneous estimation of drugs present in dosage forms UV- spectrophotometer, HPLC and HPTLC methods are considered to be most suitable. Since these are powerful and rudgged methods and also extremely precise, accurate, sensitive, specific, linear and rapid.

The drugs analysis plays an important role in the development of drugs, their manufacture and therapeutic use.Pharamaceutical industries rely upon quqntitive chemical analysis to ensure that the raw material used and the final product obtained meets the required specification.The number of drugs and drug formulations introduced into the market has beeb increasing at an alarmiing rate. These drugs or formulations may be either new entities in the market or partial structural modification of the existing drugs or novel dosage forms or multi component dosage forms.

The multi component dosage forms proved to be effective due to combined mode of action on the body.The complecity of including the precense of multiple drug entities posses considerable challenge to the analytical chemist during the development of assay procedure.The estimationof individual drugs in these multi component dosage forms becomes difficult due to some extraction or isolation procedures.

For the present study ofloxacin and ornidazole was selected. The extensive literature survey carried out revealed that there is no method reported for the simultaneous estimation of these drugs, some mathods for estimation of combined drugs by HPLC and spectrophotametry are available.Hence present study aim to developing a specific, precise, accuracy, linear, simple,

rapid, validated and cost effetive RP-HLPC method for the simultaneous estimation of these drugs in copmbined dosage forms.

The core of project for RP-HLPC amaethod was designed as follows:

1. Selection of suitable wavelength,
2. Selection of stationary phase and mobile phase,
3. Selection of initial separation conditions,
4. Optimization of chromatrophic conditions,
5. Estimation of ofloxacin and ornidazole
6. Method validation.

MATERIALS AND METHODS

MATERIALS USED FOR METHOD DEVELOPMENT AND VALIDATION

INSTRUMENT USED:

HPLC	:	Equipped with a UV detector capable of operating in the range of 200 -400 nm Shimadzu. (Software Autochrome 3000)
pH Meter	:	Range from 0 -14.Deep vision
Analytical balance	:	Accurate to 0.001g- Shimadzu
Solvent filteration Unit	:	Milli pore –Rankem
Syringe filters	:	Nylon 0.22µ filter, PVDF 0.45 µ filters
Centrifuge	:	Remi
Sonicator	:	PCI Analytical labs

APPARATUS USED:

Graduated cylinder	:	50ml, 100, and 1000ml – Borosil.
Volumetric flask	:	200ml, 100ml, 50ml –Borosil.
Volumetric pipettes	:	10ml, 5ml, 30ml, 25ml, 20ml –Borosil.
Graduate pipettes	:	5ml, 10ml, 1ml, 2ml –Borosil

Shimadzu HPLC –Model –LC 10ATvp

Specification	
Column	Cosmosil C_{18} MS –II Packed column(150mm x 4.6mm,5µm)
Pump	LC 10AT vp
Detector	UV
Temperature	Ambient

Chemicals Used:

Potassium dihydrogen ortho Phosphate : Ranbaxy

Ortho phosphoric acid : S.D fine Chem

Water : Milli Q (Ranbaxy)

Acetonitrile : Merck HPLC grade

Software Used : Auto chrome 3000

Standard Used

Ofloxacin Working Standard of Known potency

Ornidazole Working Standard of Known Potency

Source of drugs:

Samples were collected from Viencee pharma science.

METHOD DEVELOPMENT

Determination of λ max

Weighed and transferred about 100mg of ofloxacin working standard into a 100ml volumetric flask, added about 60ml of mobile phase and sonicated to dissolve and dilute to volume with mobile phase and mixed.

Transfer 1.0ml of the above solution into a 100ml volumetric flask, diluted to volume with mobile phase and mixed.

Weighed and transferred about 100mg of Ornidazole working standard into a 100ml volumetric flask, added about 60ml of mobile phase and sonicated to dissolve and dilute to volume with mobile phase and mixed.

Transfer 1.0ml of the above solution into a 100ml volumetric flask, diluted to volume with mobile phase and mixed.

Above solution scanned in the range of 200nm to 400nm

Observation:

The two absorbance graph overlain spectra it was found that the two drugs have marked absorbance at 308 nm and can be effectively used for estimation of two drugs without interference.(Figure:6)

Conclusion:

Interest of wave length concluded as 308nm

The objective of this experiment was to optimize the assay method for estimation of ofloxacin and Ornidazole based on the literature survey and the trails made. The trials mentioned below describes how the optimization was done.

Trial-1:

Buffer preparation	**:**	Weigh accurately 0.2721gms of potassium dihydrogen Orthophosphate and dissolve it in 1000ml of HPLC water. Filter through 0.45µm membrane filters and degas.
Mobile Phase	**:**	Buffer and acetonitrile were mixed in the ratio of 70:30 and Sonicated to degas filter 0.45 µm membrane filters.
Diluents	**:**	Mobile phase
Chromatographic condition	**:**	
Column	**:**	CosmosilC_{18} –MS-II column (150 x 4.6mm, 5µm Particle size)
Column Temperature	**:**	Ambient
Flow rate	**:**	1.0ml/min
Injection volume	**:**	20µl
Detector wave length	**:**	308nm
Run time	**:**	10 min
Conclusion	**:**	The peak was observed but the plate count for Ofloxacin is below 2000. (Figure:7)

Trial 2:

Buffer preparation	**:**	Weigh accurately 0.2721gms of potassium Dihydrogen Orthophosphate and dissolve it in 1000ml of HPLC water. Adjust the pH to 3.5 with dilute ortho phosphoric acid solution filter through 0.45µm membrane filters and degas.
Mobile phase	**:**	Buffer and acetonitrile were mixed in the ratio of 70:30 and sonicated to degas filter 0.45 µm membrane filters.
Diluents	**:**	Mobile phase

Chromatographic conditions:

Column	**:**	CosmosilC_{18} –MS-II column (150 x 4.6mm, 5µm particle size)
Column Temperature	**:**	Ambient
Flow rate	**:**	1.0ml/min
Injection volume	**:**	20µl
Detector wave length	**:**	308nm
Run time	**:**	10 min
Conclusion	**:**	The peak was observed but the plate count for Ofloxacin and ornidazole is below 2000. (Figure: 8)

Trial 3:

Buffer preparation	**:**	Weigh accurately 0.2721gms of Potassium dihydrogen orthophosphate and dissolve it in 1000ml of HPLC water. Adjust the pH to 3.0 with dilute ortho phosphoric acid solution filter through 0.45µm membrane filters and degas.
Mobile phase	**:**	Buffer and acetonitrile were mixed in the ratio of 70:30 and sonicated to degas filter 0.45 µm membrane filters.
Diluents	**:**	Mobile phase
Chromatographic conditions	**:**	
Column	**:**	CosmosilC_{18} –MS-II column (150 x 4.6mm, 5µm particle size)
Column Temperature	**:**	Ambient
Flow rate	**:**	1.0ml/min
Injection volume	**:**	20µl
Detector wave length	**:**	308nm
Run time	**:**	10 min
Conclusion	**:**	The peak was observed but the plate count for Ofloxacin is below 2000. (Figure: 9)

Trial 4:

Buffer Preparation	**:**	Weigh accurately 2.721gms of potassium

dihydrogen Orthophosphate and dissolve it in 1000ml of HPLC water. Adjust the pH to 3.0 with dilute orthophosphoric acid solution filter through 0.45µm membrane filters and degas.

Mobile phase : Buffer and acetonitrile were mixed in the ratio Of 70:30 and sonicated to degas filter 0.45 µm membrane filters.

Diluents : Mobile phase

Chromatographic conditions:

Column : CosmosilC_{18} –MS-II column (150 x 4.6mm, 5µm particle size)

Column Temperature : Ambient

Flow rate : 1.0ml/min

Injection volume : 20µl

Detector wave length : 308nm

Run time : 10 min

Conclusion : The peak was observed but the plate count for ofloxacin is below 2000. (Figure:10)

Trial 5:

Buffer preparation : Weigh accurately 2.721gms of potassium

dihydrogen Orthophosphate and dissolve it in 1000ml of HPLC water. Adjust the pH to 3.0 with dilute orthophosphoric acid solution filter through 0.45µm membrane filters and degas

Mobile phase **:** Buffer and acetonitrile were mixed in the ratio of 80:20 and sonicated to degas filter 0.45 µm membrane filters.

Diluents **:** Mobile phase

Chromatographic conditions:

Column **:** CosmosilC_{18} – MS-II column (150 x 4.6mm,5µm particle size)

Column Temperature **:** Ambient

Flow rate **:** 1.0ml/min

Injection volume **:** 20µl

Detector wave length **:** 308nm

Run time **:** 10 min

Conclusion **:** The peak was observed with good tailing and good shape, with plate count for ofloxacin and ornidazole 2236, 8794 and tailing factor 1.7 &1.3 and this Method was finalized for assay of ofloxacin and ornidazole tablets. (Figure: 11)

Preparation of standard solution:

Weigh and transfer accurately about 100.8mg of ofloxacin working standard and 250 .2mg of ornidazole working standard into a 100ml volumetric flask, add about 60ml of mobile phase and sonicated to dissolve and dilute to volume with mobile phase and mix.(Stock solution-I)

Transfer 10ml of the above (stock solution-I) into a 100 volumetric flask and dilute to volume with mobile phase and mix. (Stock solution -II)

Transfer 30ml of the above (stock solution-II) into a 100 volumetric flask and dilute to volume with mobile phase and mix. (Stock solution -III)

Preparation of sample solution:

Accurately weigh and transfer tablet content to about 508.2mg of sample into a 100ml volumetric flask, add about 60ml of mobile phase and sonicate for 30 minutes and make up to 100ml with mobile phase. (Stock solution-I)

Transfer 10ml of the above solution (Stock solution-I) into a 100 volumetric flask and Dilute to volume with mobile phase and mix. (Stock solution -II)

Transfer 30ml of the above solution (stock solution-II) into a 100 volumetric flask and Dilute to volume with mobile phase and mix. (Stock solution -III)

Procedure:

Equilibrate the column with mobile phase for not less than 30 minutes at a flow rate of 1.0ml/min. Separately inject 20µl of diluents as blank standard solution (six times) and sample solution into the chromatographic system. Record the chromatograms and measure the peak responses.

System suitability: The column efficiency as determined for the ofloxacin and ornidazole peak from standard solution is NLT 2000 theoretical plates**.**

Tailing factor for ofloxacin and ornidazole peak obtained from standard chromatogram should be NMT 2.0 The % RSD for the ofloxacin and ornidazole peak for 6replicate injections of standard solution should be NMT 2

The retention time of ofloxacin peak is about 3.0 minutes and ornidazole peak is about 6.0minutes

Calculations:

Quantity of ofloxacin and ornidazole present in the tablet as % of labeled amount:

$$= \frac{A_T \times W_s \times 10 \times 30 \times 100 \times 100 \times 100 \times P \times T \times 100}{A_{sT} \times 100 \times 100 \times 100 \times W_T \times 10 \times 30 \times 100 \times L}$$

AT: - Area of ofloxacin peak in sample solution

AT: - Area of ornidazole peak in sample solution

As: - Average area of ofloxacin peak obtained from six replicate injections of standard solution.

As: - Average area of Ornidazole peak obtained from six replicate injections of standard solution.

P: - Purity of ofloxacin working standard used

P: - Purity of ornidazole working standard used

W_T: - Weight of sample taken, in mg

Ws: - Weight of ofloxacin working standard taken, in mg

Ws: - Weight of ornidazole working standard taken,in mg

L: - Labeled amount of ofloxacin, in mg per tablet

L: - Labeled amount of ornidazole, in mg per tablet

T: - Average fill weight of tablet in mg.

METHOD VALIDATION

1. System suitability and system precision

A standard solution was prepared by using ofloxacin and ornidazole working Standards as per test method and was injected six times into the HPLC system.

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from six replicate injections for ofloxacin and ornidazole retention times and peak areas.

Acceptance criteria:

1. The % RSD for the retention times of principal peak from six replicate injections of each standard solution should be not more than 1.0 and for peak area should not be More than 2.0.
2. The number of theoretical plates (N) for the ofloxacin peak and ornidazole peak is NLT 2000.
3. Tailing factor (T) for the ofloxacin peak and ornidazole peak is NMT 2.0

Table: 1 system suitability for ofloxacin

Injection	Retention time	Peak Area	USP plate count	USP Tailing
1	3.0	1105	2696	1.8
2	3.0	1108	2687	1.8
3	3.0	1108	2522	1.8
4	3.0	1106	2189	1.7
5	3.0	1104	2539	1.8
6	3.0	1112	2494	1.8
Mean	3.0	1107	2521	1.78
SD	2.60			
% RSD	0.24			

Observation:

The %RSD for retention times and peak areas were found to be within the limits. (Figure: 12-17)

Table: 2 system suitability for ornidazole

Injection	Retention time	Peak Area	USP plate count	USP Tailing
1	6.0	1810	7453	1.3
2	6.0	1815	7460	1.3
3	6.0	1804	7511	1.3
4	6.0	1819	7531	1.3
5	6.0	1813	7495	1.3
6	6.0	1792	7501	1.3
Mean	6.0	1809	7492	1.3
SD	8.82			
% RSD	0.49			

Observation:

The %RSD for retention times and peak areas were found to be within the limits. (Figure: 13-18)

Accuracy: (Recovery) A study of accuracy was conducted. Drug assay was performed in triplicate as per test method by spiking the ofloxacin and ornidazole drug substance to the placebo equivalent to 50%, 100%, and 150% of the labeled amount as per the test method. The average % recovery of ofloxacin and ornidazole was calculated.

Separately inject the blank, placebo, ofloxacin and ornidazole in to the chromatograph.

Acceptance criteria:

The mean % recovery of ofloxacin and ornidazole at each level should be not less than 98.0 % and not more than 102.0%

Table: 3 Accuracy for ofloxacin

Concentration % of spiked level	**Amount added (in mg as ofloxacin)**	**Amount found (in mg as ofloxacin)**	**% Recovery**	**Statistical analysis of % Recovery**	
50% Sample 1	100.7	99.3	98.6	MEAN	99.1
50% Sample 2	100.8	100.3	99.5	SD	0.37
50% Sample 3	101.3	100.5	99.2	%RSD	0.38
100% Sample 1	200.7	198.9	98.2	MEAN	98.9
100% Sample 2	200.8	199.7	98.9	SD	0.53
100% Sample 3	200.3	199.8	99.5	%RSD	0.54
150% Sample 1	300.5	299.8	99.3	MEAN	99.1
150% Sample 2	300.7	299.9	98.5	SD	0.43
150% Sample 3	301.4	300.9	99.5	%RSD	0.44

Observation: The recovery results indicating that the test method has an acceptable level of accuracy. (Figure: 19-27)

Table: 4 Accuracy for ornidazole

Concentration % of spiked level	**Amount added (in mg as ornidazole)**	**Amount found (in mg as ornidazole)**	**% Recovery**	**Statistical analysis of % Recovery**	
50% Sample 1	250.8	249.2	98.4	MEAN	98.7
50% Sample 2	248.5	247.7	99.2	SD	0.38
50% Sample 3	250.7	249.1	98.4	%RSD	0.38
100% Sample 1	500.5	499.4	98.9	MEAN	98.5
100% Sample 2	500.2	498.3	98.1	SD	0.33
100% Sample 3	499.4	498.0	98.6	%RSD	0.33
150% Sample 1	750.4	749.1	98.8	MEAN	98.8
150% Sample 2	749.6	749.1	99.5	SD	0.54
150% Sample 3	750.2	748.4	98.2	%RSD	0.54

Observation: The recovery results indicating that the test method has an acceptable level of accuracy. (Figure: 19-27)

3. Precision:

a. System precision: standard solution was prepared as per test method and injected Six times in to chromatographic system.

b. Method precision: prepared six sample solutions of 100mg strength as per test Method and injected each solution into chromatographic system.

Acceptance criteria:

1. % RSD of % assay results from six samples should be NMT 2.0
2. Assay should be in the range of test method i.e. .not less than 90.0% and not more than 110.0%

a)System precision:

Table: 5 system precision for ofloxacin

Injection	Peak Areas	Theoretical plates	Tailing factor
1	1077	2962	1.7
2	1095	2478	1.6
3	1096	2878	1.6
4	1088	2460	1.7
5	1086	2923	1.6
6	1093	2495	1.7
Mean	1089	2699	1.65
SD	6.51		
%RSD	0.60		

Table: 6 system precision for ornidazole

	Injection	**Peak Areas**	**Theoretical plates**	**Tailing factor**
Concentration 100%	1	1767	8028	1.2
	2	1770	8035	1.2
	3	1792	9374	1.3
	4	1781	9444	1.2
	5	1785	7971	1.3
	6	1822	9148	1.2
Statistical analysis	Mean	1786	8667	1.23
	SD	18.14		
	%RSD	1.02		

b) Method precision:

Table: 7 Method precision for ofloxacin

Sample No.	**Injection No.**	**Area**	**Average Area**	**% Assay**
1	1	1144	1134	100.9
	2	1124		
2	1	1127	1136	100.7
	2	1145		
3	1	1135	1137	100.4
	2	1138		
Mean				100.7
Standard deviation				0.21
%RSD				0.20

Table: 8 Method precision for Ornidazole

Sample No.	Injection No.	Area	Average Area	% Assay
1	1	1807	1799	99.8
	2	1790		
2	1	1794	1791	99.4
	2	1787		
3	1	1796	1800	99.6
	2	1803		
Mean				99.6
Standard deviation				0.16
%RSD				0.16

Observation: The precision study has shown that the test method is precise. (Figure 34- 39)

4. Linearity of test method:

Five linearity solutions were prepared using ofloxacin and ornidazole working standard at concentration levels from 50% to 150% of target concentration of ofloxacin and ornidazole (50%,75%,100%,125% and 150%) .Measure the peak area response of solution at level 1and level 5 three times.

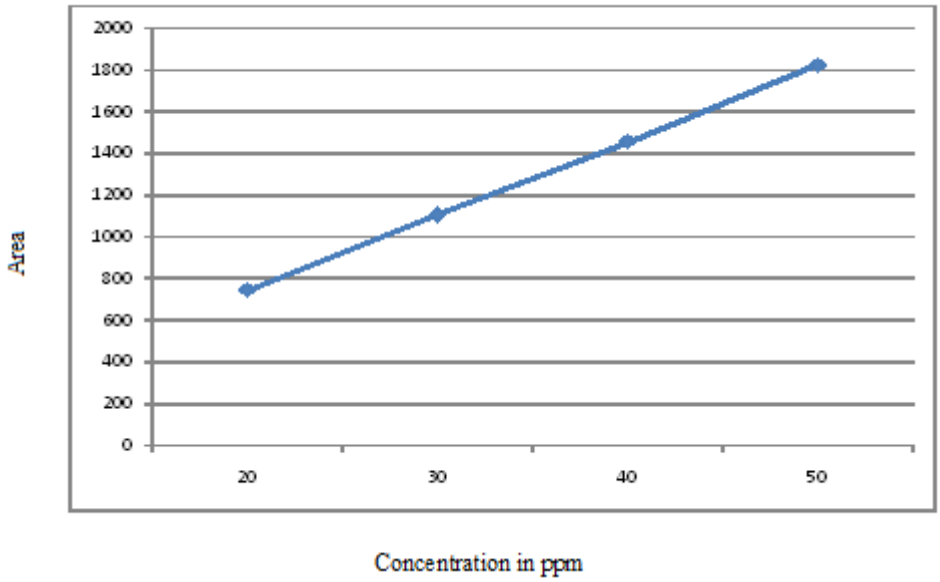
Acceptance criteria:

Correlation coefficient should be not less than 0.999.
% of y- intercept should be $\pm$ 2.0
% of RSD for level 1 and level 5 should be not more than 2.0

Table: 9 Linearity for ofloxacin

Linearity	Concentration	Average Area	%RSD	Statistical Analysis

Level	**ppm**				
L1-50%	10ppm	372	0.46	Slope	0.01
L2-75%	20ppm	744	0.23	%intercept	-1.19
L3-100%	30ppm	1108	0.26	Correlation Coefficient	0.9998
L4-125%	40ppm	1456	0.12		
L5-150%	50ppm	1824	0.16		



Observation:

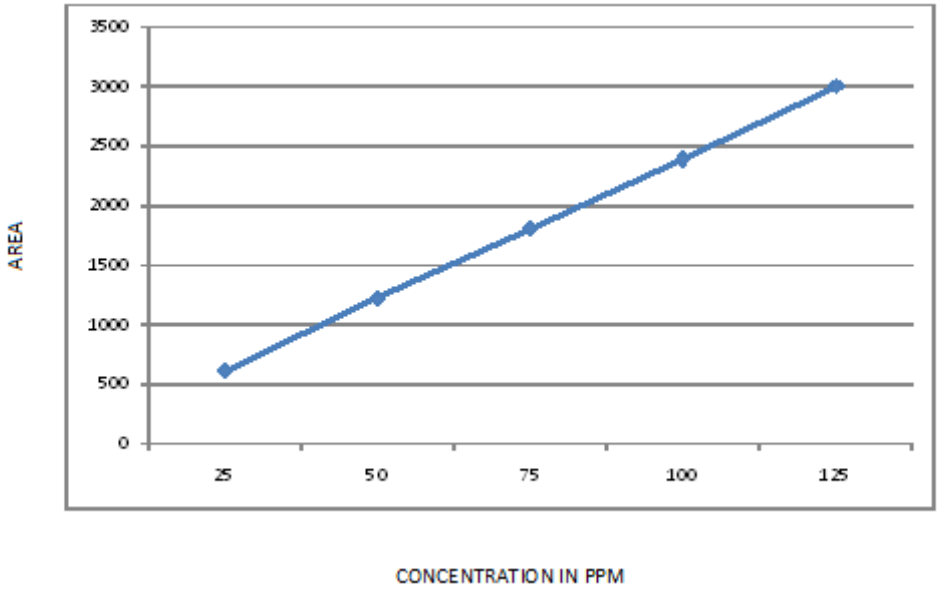
The correlation coefficient was found to be 0.9998

From the above study it was established that the linearity of test method is from 50% to 150% of the target concentration. (Figure: 40-54)

Table: 10 Linearity for ornidazole

Linearity Level	**Concentration ppm**	**Average Area**	**%RSD**	**Statistical Analysis**

L1-50%	25ppm	612	0.46	Slope	0.01
L2-75%	50ppm	1220	0.22	%intercept	-0.98
L3-100%	75ppm	1806	0.55	Correlation Coefficient	0.9999
L4-125%	100ppm	2388	0.14		
L5-150%	125ppm	3000	0.06		



Observation:

The correlation coefficient was found to be 0.9999

From the above study it was established that the linearity of test method is from 50% to 150% of the target concentration. (Figure: 40-54)

4. Range:

Data from linearity, precision, accuracy, sections was considered to establish range of the method. The results were summarized in table 24

Acceptance criteria:

For linearity, Correlation coefficient shall be ≥0.999.

For precision, %RSD of assay of 3 replicate sample preparations shall be NMT 2.0%.

For accuracy, individual recovery at each spike level should be within 98.0% to 102%

Table: 11 Range for ofloxacin

Parameter	Acceptance criteria	Result
Linearity	R≥0.999	0.9998
Precision	%RSD of 3 replicates NMT 2.0	0.20
Accuracy	Recovery 98.0% to 102.0%	99.03

Table: 12 Range for Ornidazole

Parameter	Acceptance criteria	Result
Linearity	R≥0.999	0.9999
Precision	%RSD of 3 replicates NMT 2.0	0.16
Accuracy	Recovery 98.0% to 102.0%	98.67

5. Selectivity /Specificity:

Placebo interference:

A study to establish the interference of placebo was conducted. Samples were prepared in triplicate by taking the placebo equivalent to about the weight in portion of test preparation as per the test method.

Acceptance criteria:

The chromatogram of placebo should not show any peak at the retention time of ofloxacin and ornidazole.

Table: 13 placebo interference

Ofloxacin	Ornidazole	Peak found
1	1	No
2	2	No
3	3	No

Observation: From placebo chromatograms, it was concluded that there was no interference with placebo as no peaks were observed at the retention times of ofloxacin and ornidazole peaks.

6. Limit of detection and limit of quantification:

Procedure:

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be determined quantitatively with suitable precision and accuracy. The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact Value.

As per linearity curve, we have prepared 1 ppm for detection of ofloxacin and ornidazole, 3ppm for quantitation for ofloxacin and ornidazole. Determine the signal noise ratio about 3.Determine the ratio at analyte concentration of 0.001%.If signal noise is 10 or more, quality this concentration as limit of quantification .if signal noise ratio is less than 10,increase the concentration but not beyond 0.003%,to achieve the signal to noise ratio 10 and then qualify the concentration as limit of quantification.

7. Ruggedness of the test method:

System to system /Analyst to analyst /column to column variability:

System to system /analyst to analyst /column to column variability study was conducted on different HPLC system, different columns and different analysts under similar conditions at different times. Six samples were prepared and each were analyzed as per test method. The relative standard deviation for ofloxacin and ornidazole was found to be below 2% on the columns, system and analysts.

Comparison of both the results obtained on two different HPLC systems, different column and different analysts shows that the assay test method is rugged for system to system /analyst to analyst/column to column variability.

Acceptance criteria:

The % RSD of ofloxacin and ornidazole from the six sample preparations should be not more than 2%

Table – 14 Ruggedness for ofloxacin

S.No	Concentration for 30ppm	Average area of six samples	%RSD
1	Analyst -1	1089	0.60
2	Analyst -2	1107	0.24

Table – 15 Ruggedness for ornidazole

S.No	Concentration for 75 ppm	Average area of six samples	%RSD
1	Analyst -1	1789	1.02
2	Analyst -2	1809	0.49

Observation: The % RSD was found within the limits.

8. Robustness:

i) Filter variability: (At least two filters).

For demonstrating that the filtration does not affect the analysis results. At least two types of filters were validated before use. Prepare the standard solution as per test method. Filter the standard solution through individual filter .PVDF and nylon filter were used. Inject unfiltered standard solution and filtered standard solution into the HPLC system under the test condition.

Calculate the average area of duplicate injections of individual standard solutions and determine similarity factor of filtered standard solution against unfiltered standard.

Similarity factor = A/B

Where, A: Average area of filtered standard

B: Average area of unfiltered standard

Acceptance criteria:

The similarity factor of test solution against unfiltered standard solution should be in the range of 0.98 to 1.02.

Table: 16 Filter validations for ofloxacin

S.No	Preparation of sample (ofloxacin)	Standard area	Sample area	Average Similarity Factor
1	By centrifugation	1089	1086	0.989
2			1068	
1	By 0.45µ PVDF Filter		1070	0.990
2			1087	
1	By Nylon 0.22 µ Filter		1075	0.989
2			1078	

Table: 17 Filter validations for ornidazole

S.No	Preparation of sample (ofloxacin)	Standard Area	Sample Area	Average Similarity Factor
1	By centrifugation	1786	1789	0.996
2			1768	
1	By 0.45µ PVDF Filter		1718	0.990
2			1712	
1	By Nylon 0.22 µ Filter		1734	0.992
2			1715	

Observation: The similarity factor was found to be within the limits.

ii) Effect of variation in mobile phase composition:

A study was conducted to determine the effect of variation in organic phase composition in mobile phase. Standard solution prepared as per the test method was injected into the HPLC system with 90% and 110% of organic phase with respect to test method. The system suitability parameters were evaluated and found to be within the limits for both compositions. Ofloxacin and ornidazole was resolved from all

other peaks and the retention times were comparable with those obtained for mobile phase having 100% of the organic phase. From the study it was established that the allowable variation in mobile phase Composition is 90% to 110% of the method highest organic phase of mobile phase.

Acceptance criteria:

The Tailing factor for ofloxacin and ornidazole standards should be NMT 2.0 for variation in organic phase

Table: 18 Effect of variable in mobile phase composition

S.No	Mobile phase composition (buffer:ACN)	Area of ofloxacin	%RSD of peak area	Tailing factor
1.	87:13	1075	0.93	1.3
2.	85:15	1103	0.36	1.4
3.	83:17	1131	0.40	1.5

Table: 19 Effect of variable in mobile phase composition

S.No	Mobile phase composition (buffer:ACN)	Area of ornidazole	%RSD of peak area	Tailing factor
1.	87:13	1735	0.58	1.0
2.	85:15	1758	0.20	1.1
3.	83:17	1771	0.11	1.2

Observation:

The tailing factor for ofloxacin and ornidazole is found to be within the limits

iii) Effect of variation of flow rate :

A study was conducted to determine the effect of variation in flow rate. standard solution prepared as per the test method was injected into the HPLC system using flow rates, 0.9ml/min and 1.1ml/min.The system suitability parameters were

evaluated and found to be within the limits for 0.9ml/min and 1.1ml/min flow.

ofloxacin and ornidazole was resolved from all other peaks and retention time were comparable with those obtained for mobile phase flow rate 1.0ml/min.

from the above study it was established that the allowable variation in flow rates is 0.9ml/min and 1.1ml/ml.

Acceptance criteria:

The tailing factor of ofloxacin and ornidazole standards should be NMT 2.0 for variation in flow.

Table -20 Effect of variation in flow rate

S.No	Flow rate	Area of ofloxacin	%RSD of the peak area	Tailing factor
1	0.9ml/min	1208	0.21	1.7
2	1.0ml/min	1089	0.60	1.7
3	1.1ml/min	970	0.52	1.7

Table -21 Effect of variation in flow rate

S.No	Flow rate	Area of ornidazole	%RSD of the peak area	Tailing factor
1	0.9ml/min	1987	0.60	1.3
2	1.0ml/min	1786	1.02	1.2
3	1.1ml/min	1628	0.15	1.2

Observation:

The tailing factor for ofloxacin and ornidazole is found to be within the limits**.**

iv) Effect of variation of pH:

A study was conducted to determine the effect of variation in pH. Standard and sample solutions were prepared as per the test method and inject into the HPLC system using pH 2.8 and 3.2 .The system suitability parameters were evaluated and found to be within the limits for pH 2.8 and 3.2

Ofloxacin was resolved from all other peaks and the retention times were comparable with those obtained for mobile phase having pH 3.0.

From the above study it was established that the allowable variation in pH 2.8 and 3.2

Acceptance criteria:

The tailing factor of ofloxacin and ornidazole standard should be NMT 2.0 for variation in pH.

Table -22 Effect of pH

S.No	pH	Area of ofloxacin	%RSD of peak area	Tailing factor
1	**2.8**	**1078**	**0.74**	**1.4**
2	**3.0**	**1089**	**0.60**	**1.7**
3	**3.2**	**1070**	**0.51**	**1.5**

Table -23 Effect of pH

S.No	pH	Area of ornidazole	%RSD of peak area	Tailing factor
1	**2.8**	**1760**	**0.43**	**1.2**
2	**3.0**	**1786**	**1.02**	**1.2**
3	**3.2**	**1810**	**0.44**	**1.2**

Observation:

The tailing factor for ofloxacin and ornidazole is found to be within the limits.

Conclusion:

From the above results it is concluded that the method is robust.

CHROMATOGRAMS

UV Spectrum:

Sample Spectrum:

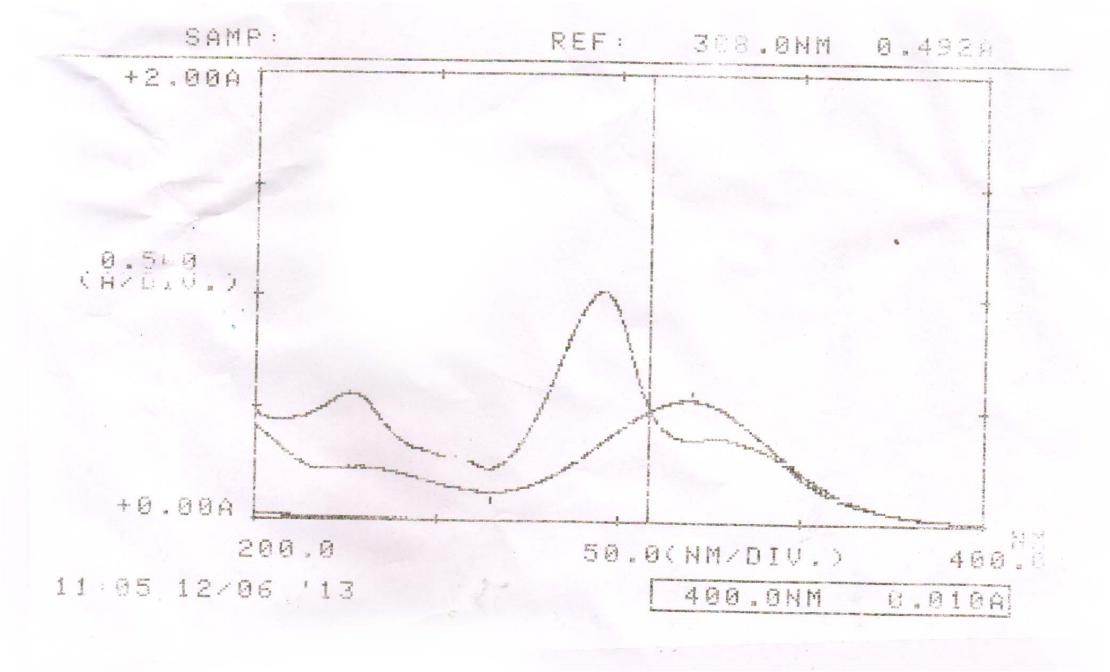
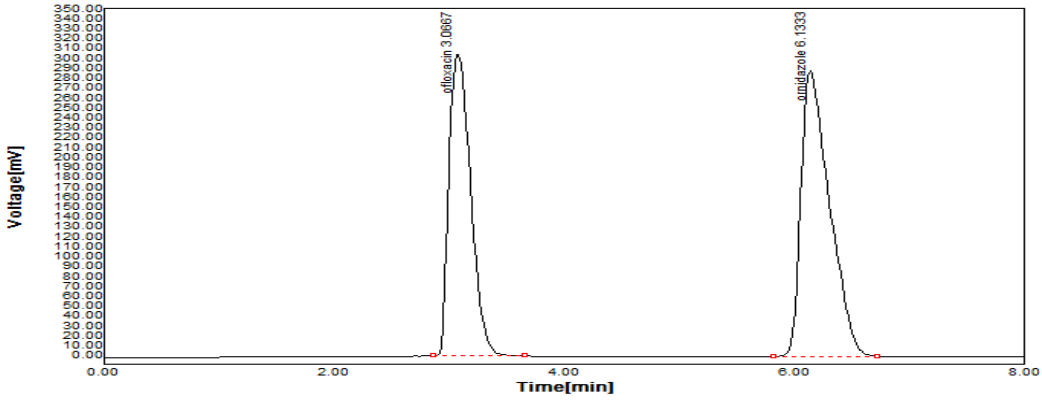


Figure: 6

Method development trails in HPLC

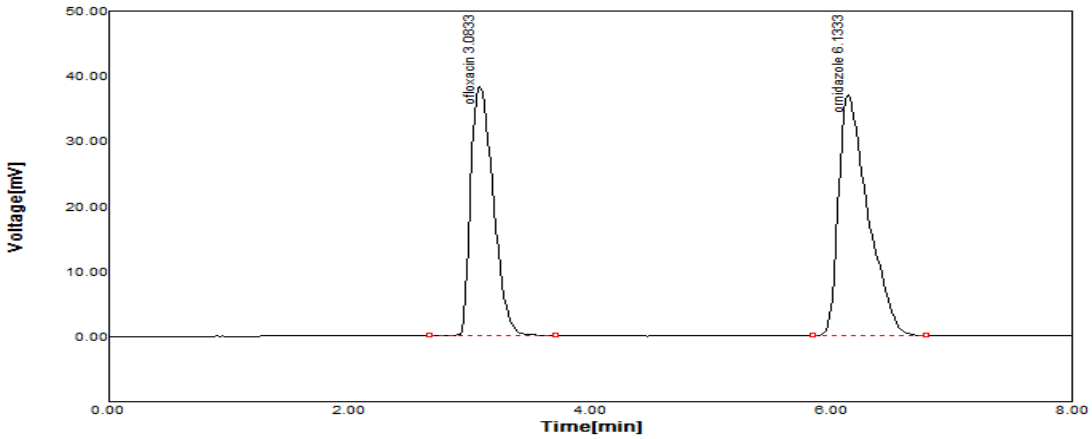
Trial No.1



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.1	3981	1333	1.6
2	ornidazole	6.1	4980	2110	1.7

Figure: 7

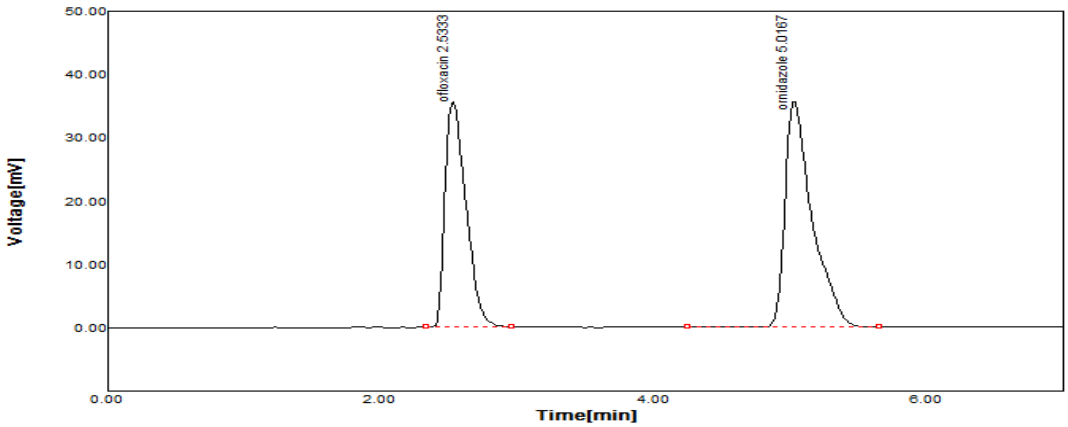
Trial No.2



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.1	496	1314	1.4
2	ornidazole	6.1	629	1838	1.7

Figure: 8

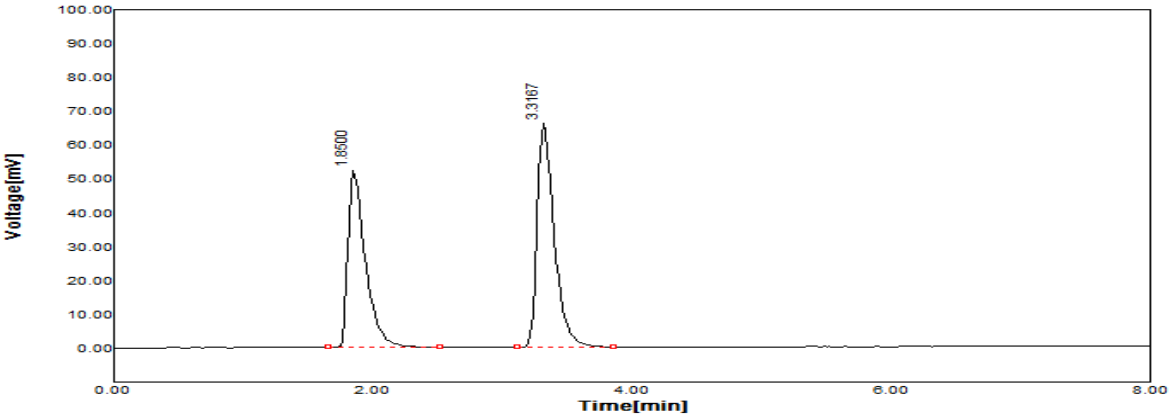
Trial No.3



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	2.5	377	1845	1.5
2	ornidazole	5.0	492	3359	1.9

Figure: 9

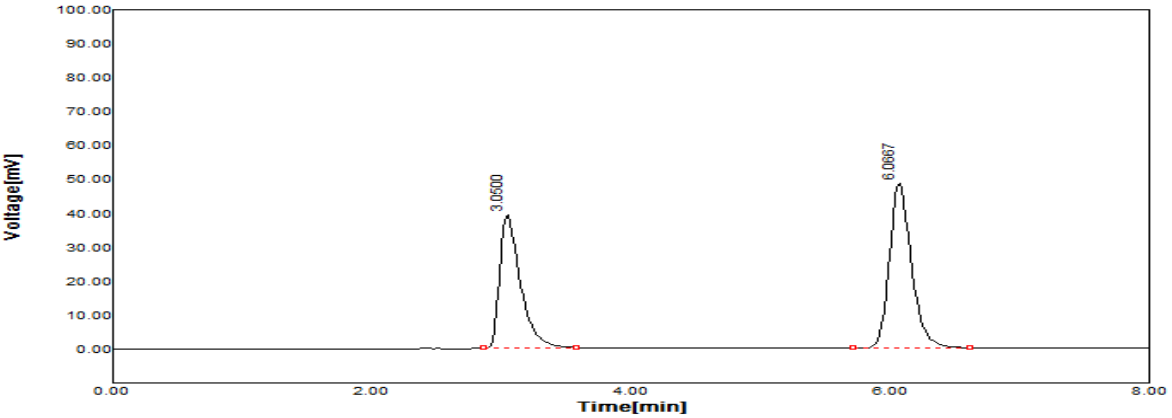
Trial No.4



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	1.9	485	1365	2.0
2	ornidazole	3.3	603	4358	1.6

Figure: 10

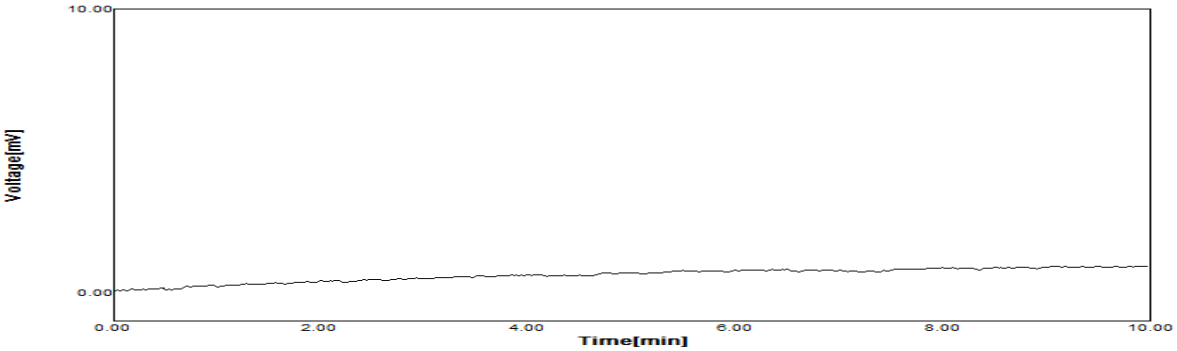
Trial No.5



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.0	429	2236	1.7
2	ornidazole	6.1	572	8794	1.3

Figure: 11

Mobile phase Blank

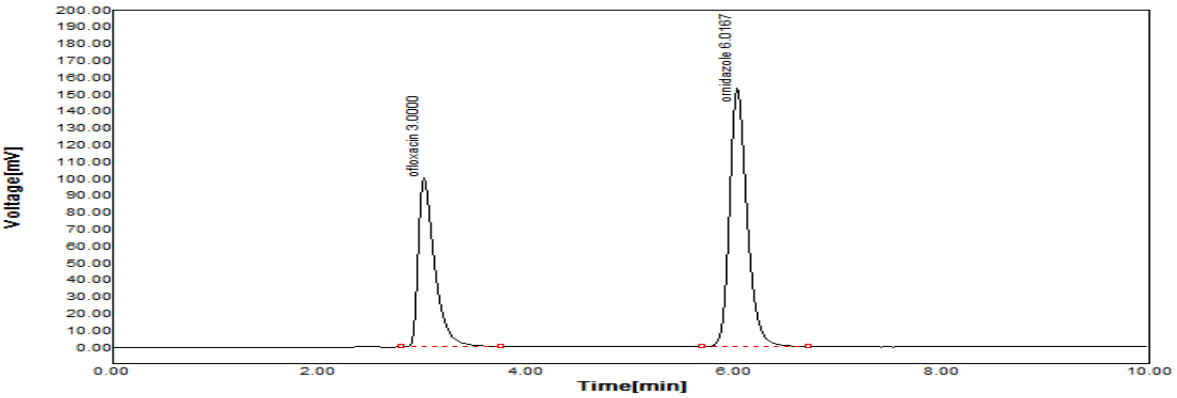


	Name	RT	Area	%Area
1	ofloxacin	3.0		
2	ornidazole	6.0		

Figure: 12

System suitability:

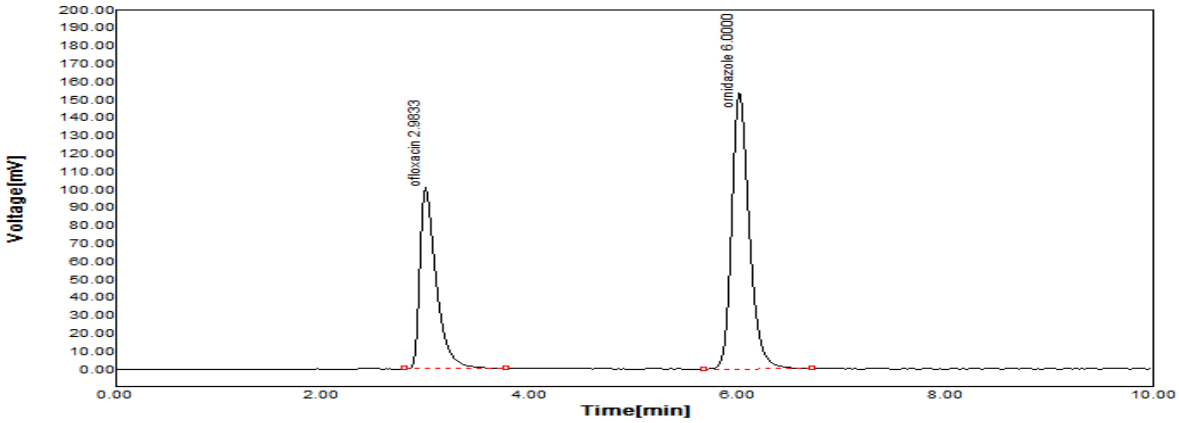
Injection: 1



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.0	1105	2696	1.8
2	ornidazole	6.0	1810	7453	1.3

Figure: 13

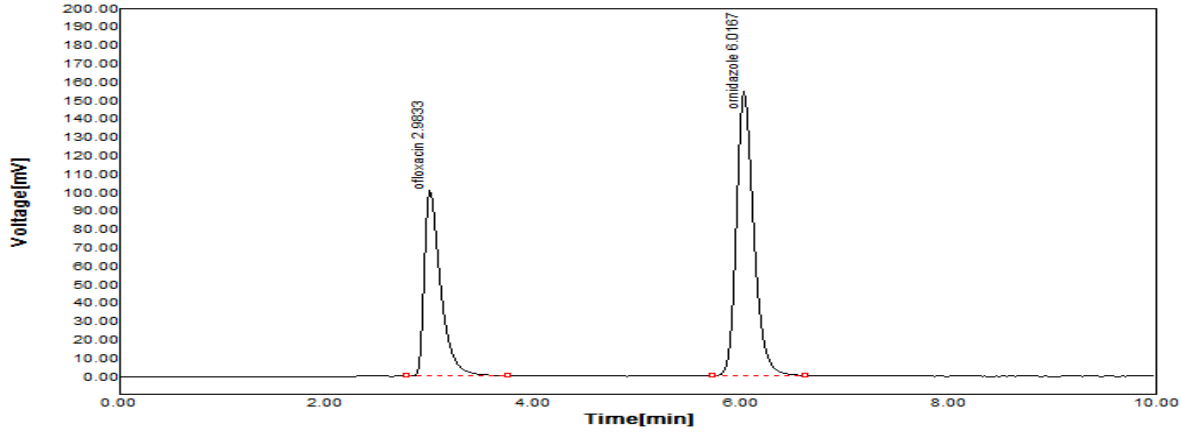
Injection: 2



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.0	1108	2687	1.8
2	ornidazole	6.0	1815	7460	1.3

Figure: 14

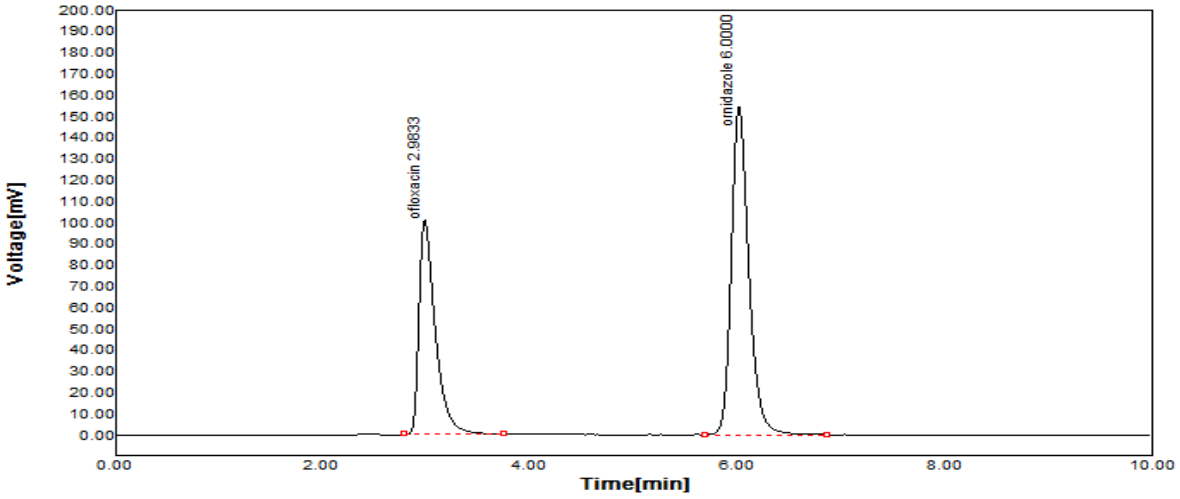
Injection: 3



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.0	1108	2522	1.8
2	ornidazole	6.0	1804	7511	1.3

Figure: 15

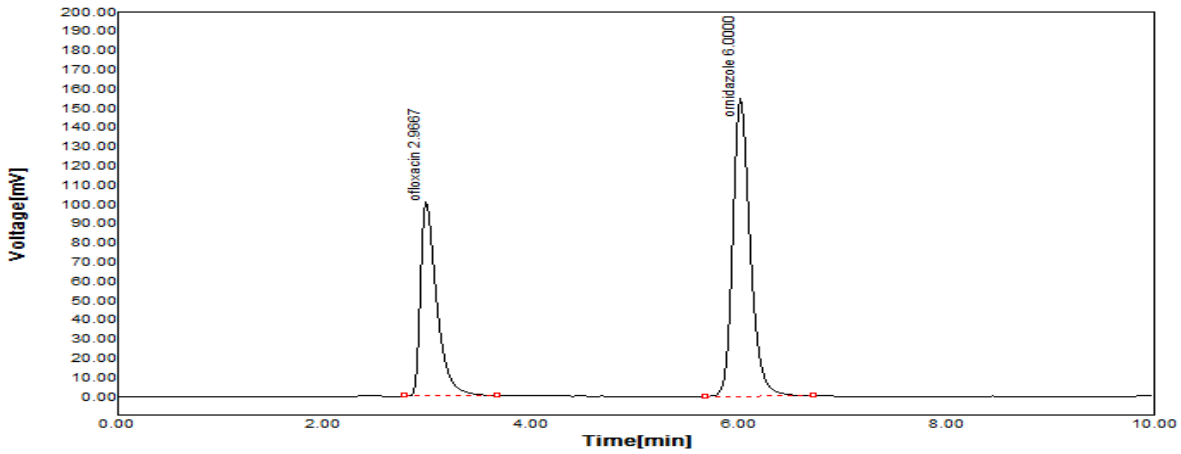
Injection: 4



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.0	1106	2189	1.7
2	ornidazole	6.0	1804	7531	1.3

Figure: 16

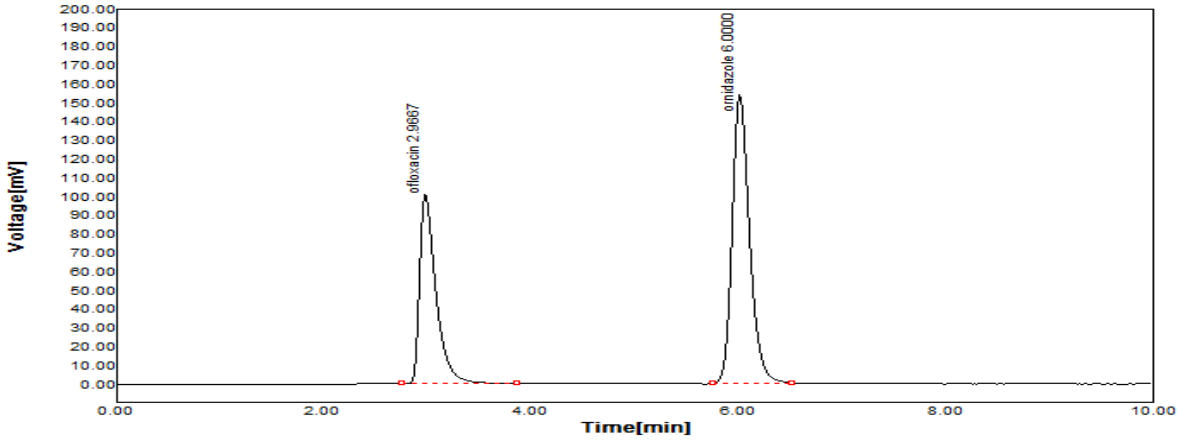
Injection: 5



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.0	1104	2539	1.8
2	ornidazole	6.0	1813	7495	1.3

Figure:17

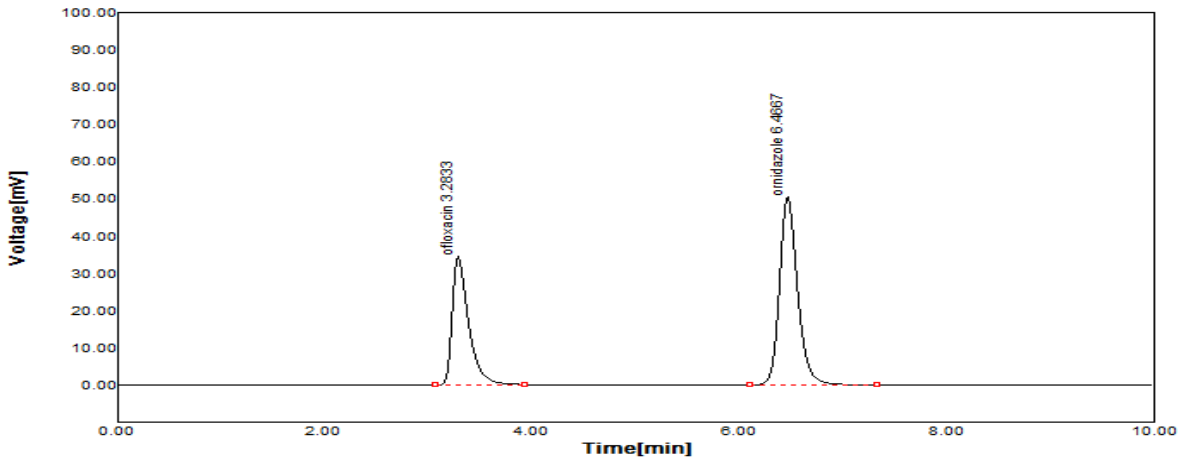
Injection: 6



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.0	1112	2494	1.8
2	ornidazole	6.0	1792	7501	1.3

Accuracy Chromatogram

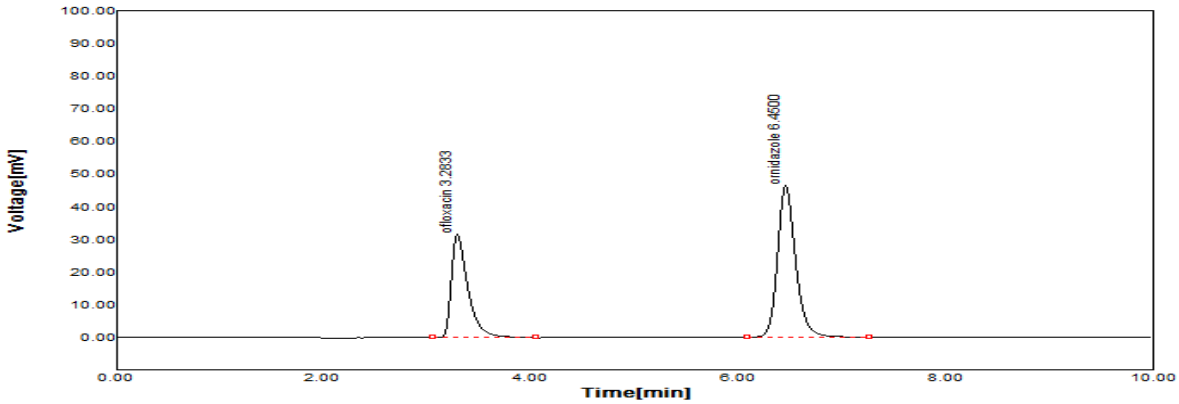
50% sample-I



	Name	RT	Area
1	ofloxacin	3.3	367
2	ornidazole	6.5	608

Figure: 19

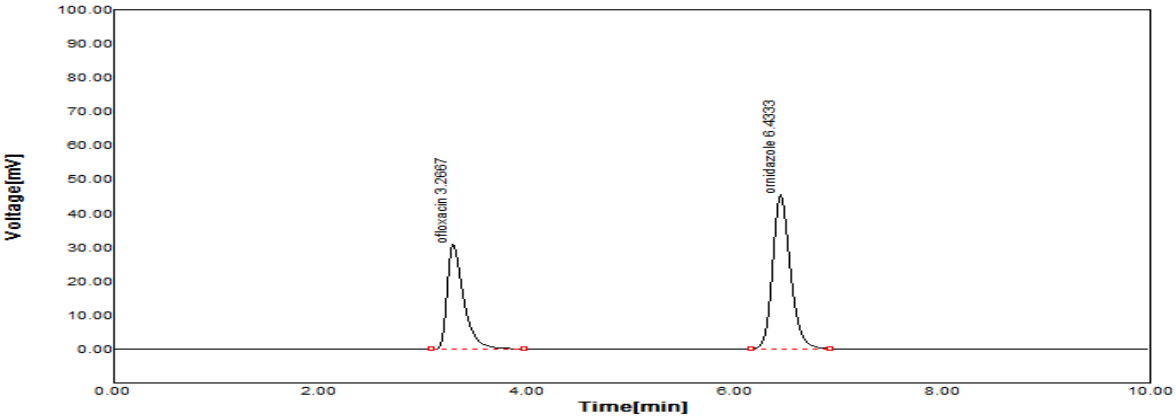
50% Sample-II



	Name	RT	Area
1	ofloxacin	3.3	370
2	ornidazole	6.5	610

Figure:20

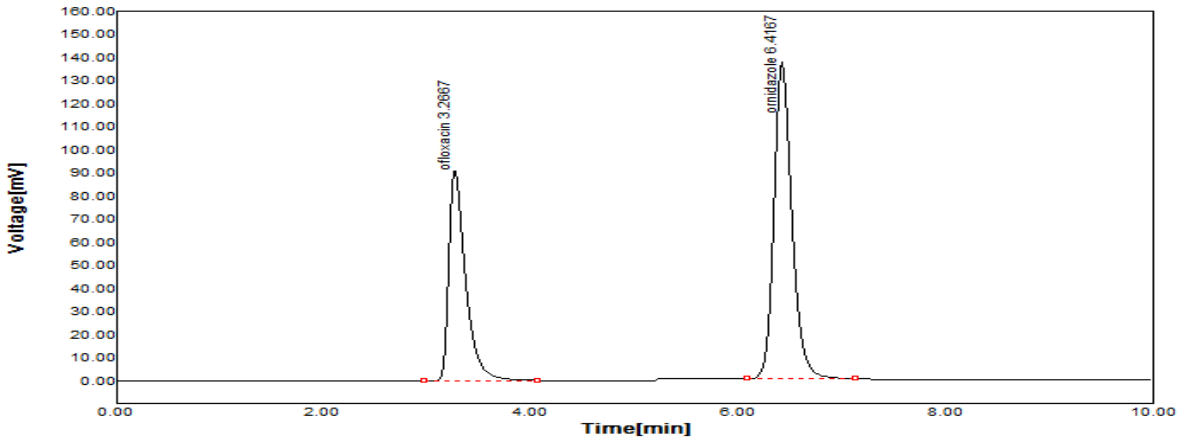
50% Sample-III



	Name	RT	Area
1	ofloxacin	3.3	369
2	ornidazole	6.4	608

Figure:21

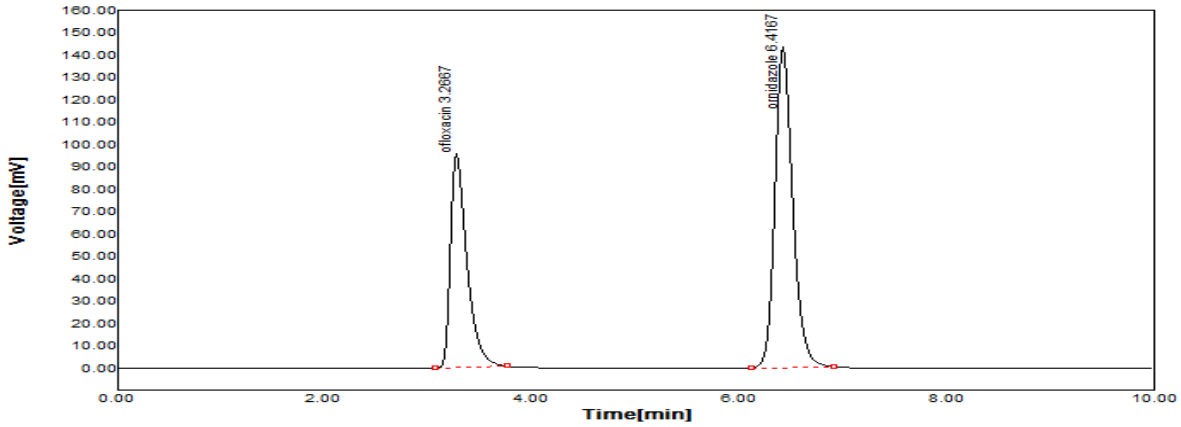
100% Sample-I



	Name	RT	Area
1	ofloxacin	3.3	1098
2	ornidazole	6.4	1802

Figure:22

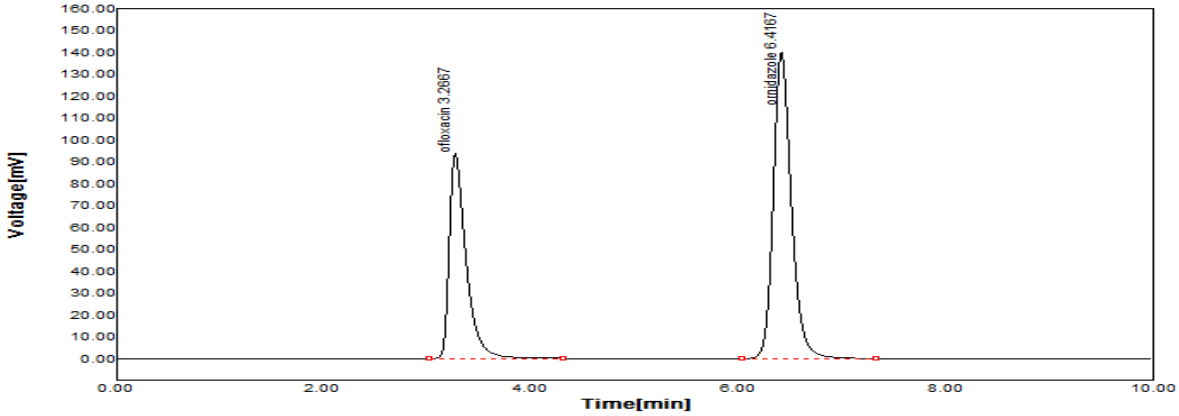
100% Sample-II



	Name	RT	Area
1	ofloxacin	3.3	1102
2	ornidazole	6.4	1799

Figure:23

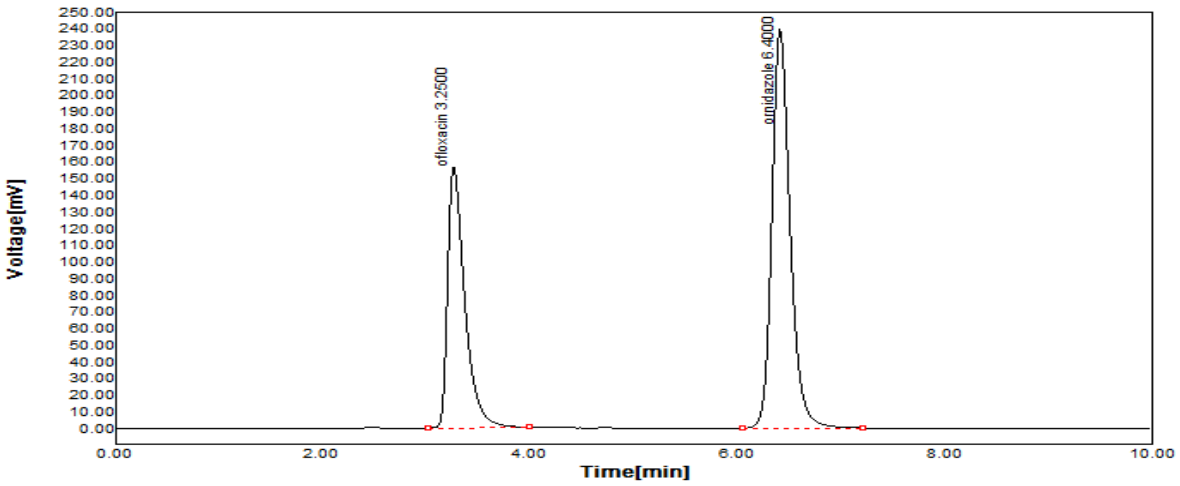
100% Sample-III



	Name	RT	Area
1	ofloxacin	3.3	1105
2	ornidazole	6.4	1819

Figure:24

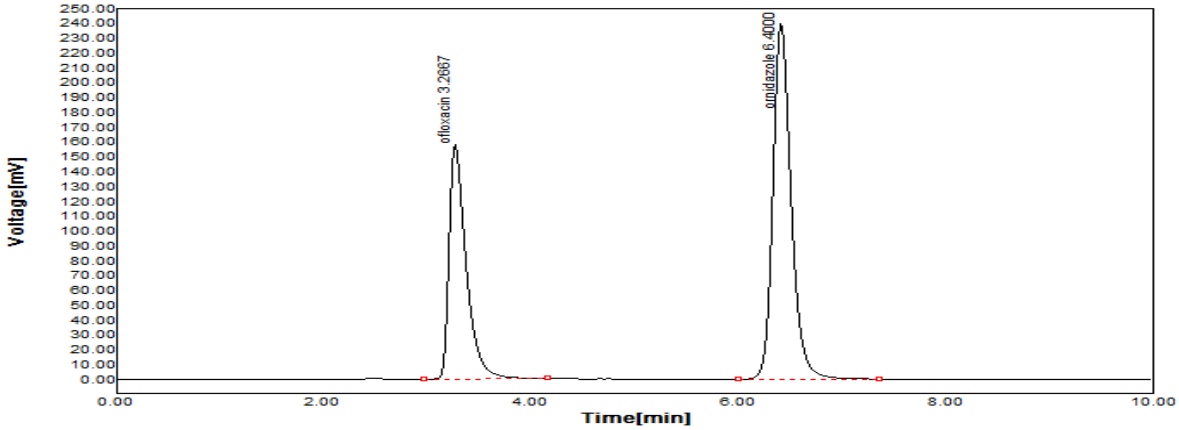
150% Sample-1



	Name	RT	Area
1	ofloxacin	3.3	1820
2	ornidazole	6.4	2995

Figure: 25

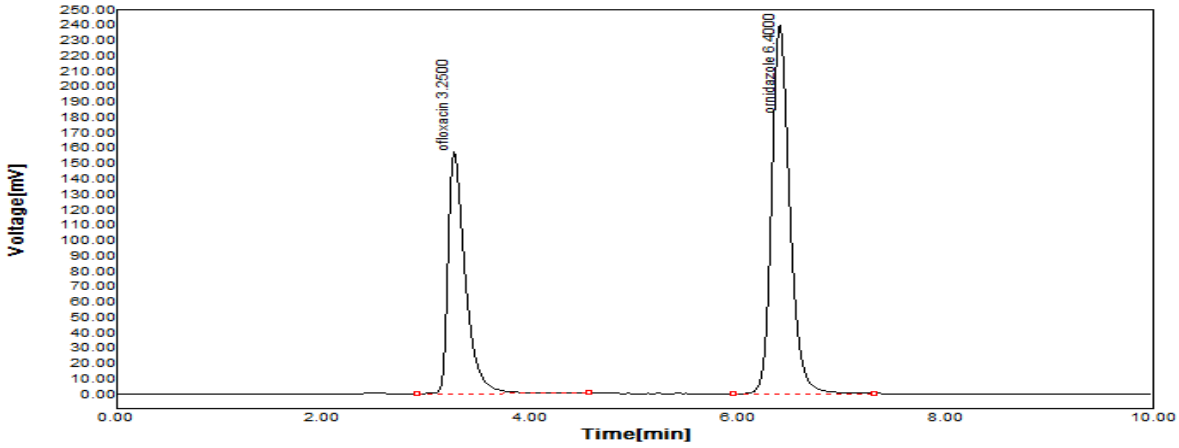
150%Sample-II



	Name	RT	Area
1	ofloxacin	3.3	1816
2	ornidazole	6.4	2998

Figure: 26

150%Sample-III

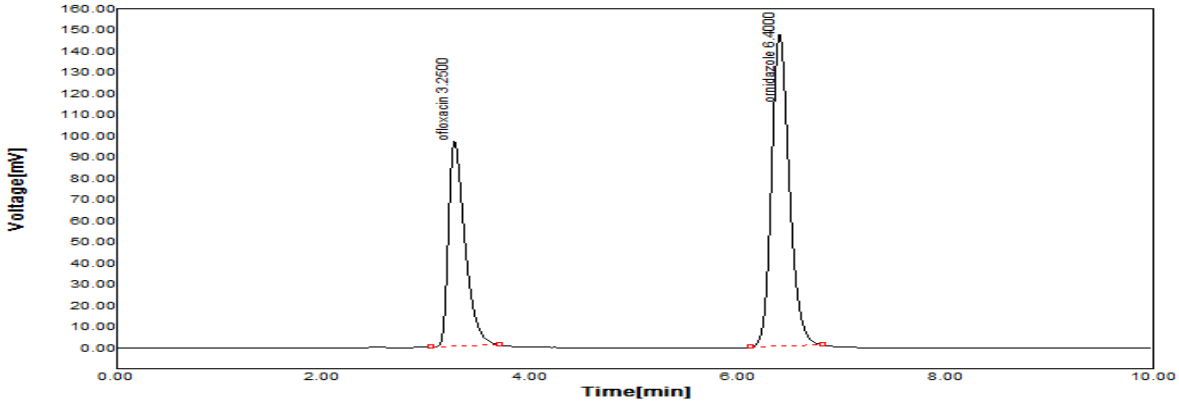


	Name	RT	Area
1	ofloxacin	3.3	1821
2	ornidazole	6.4	2993

Figure: 27

System Precision Chromatograms

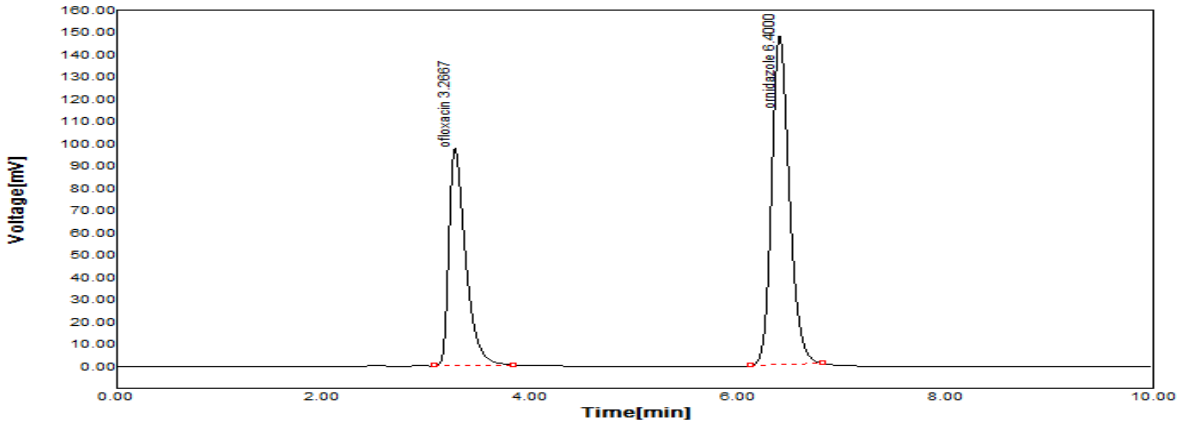
Injection-1



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.3	1077	2962	1.7
2	ornidazole	6.4	1767	8028	1.2

Figure: 28

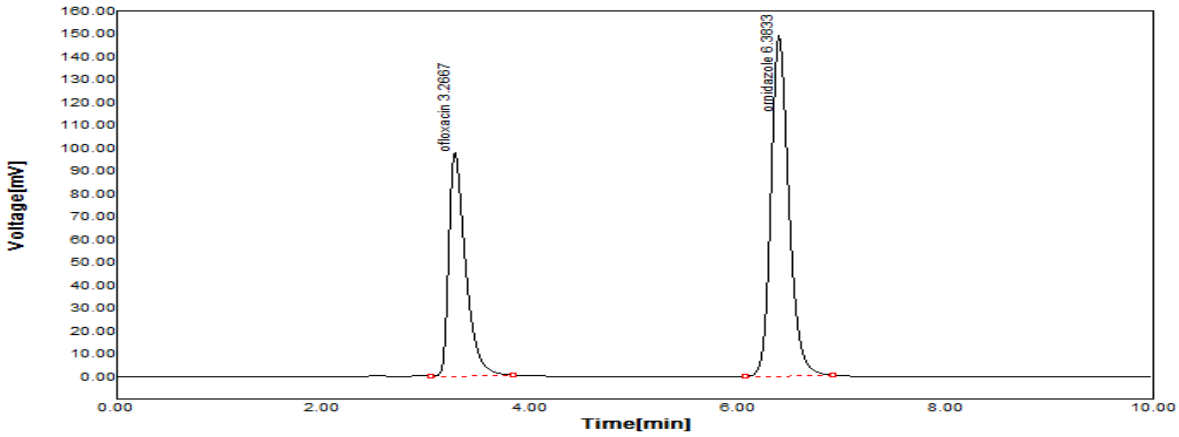
Injection-2



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.3	1095	2438	1.6
2	ornidazole	6.4	1770	8035	1.2

Figure: 29

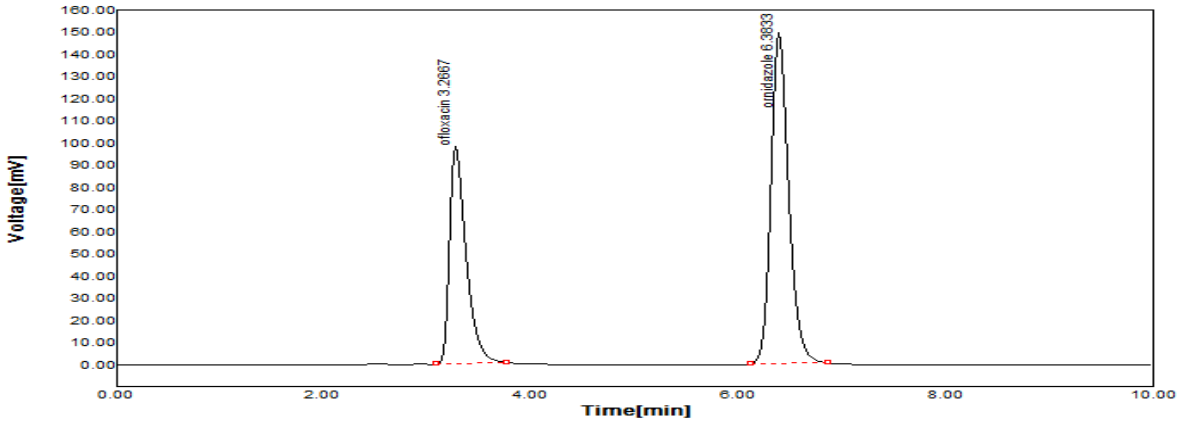
Injection-3



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.3	1096	2878	1.6
2	ornidazole	6.4	1792	9374	1.3

Figure: 30

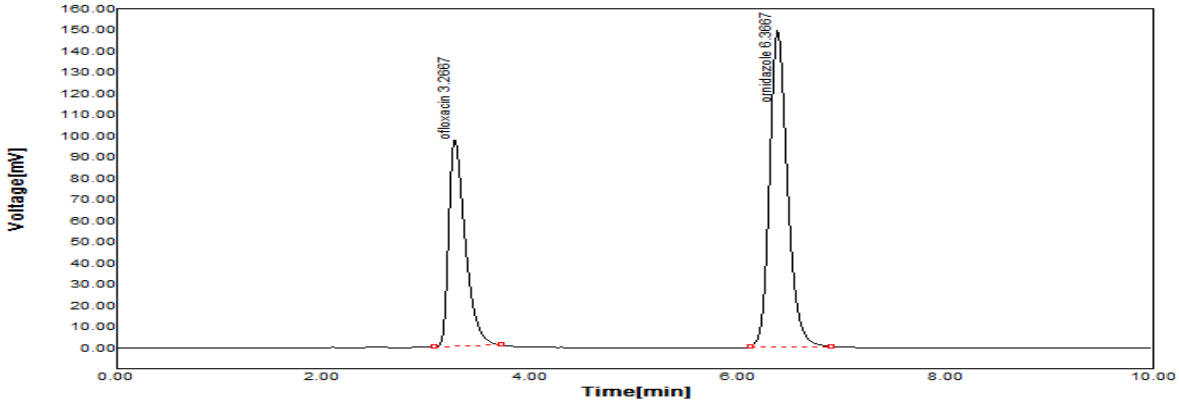
Injection-4



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.3	1088	2460	1.7
2	ornidazole	6.4	1781	9444	1.2

Figure:31

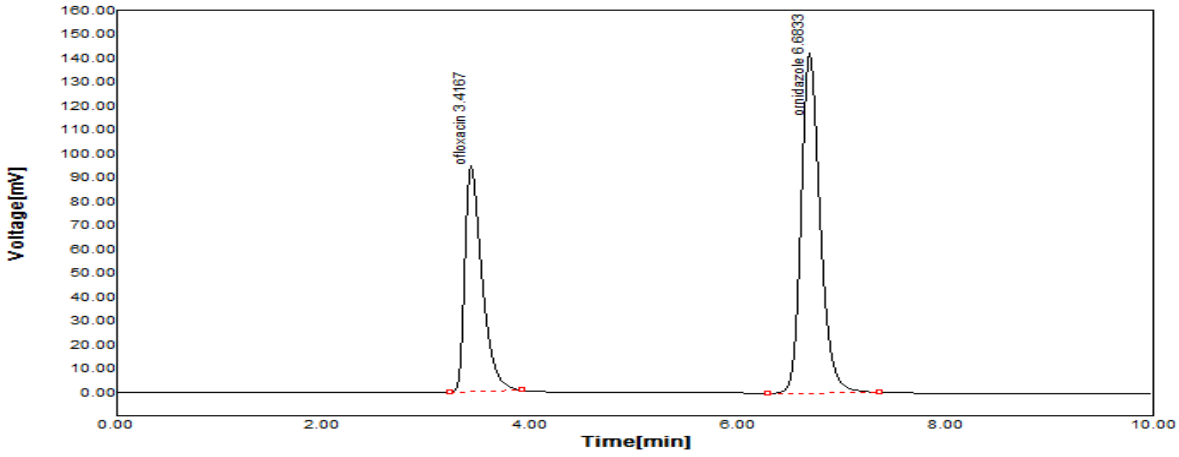
Injection-5



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.3	1086	2923	1.6
2	ornidazole	6.4	1785	7971	1.3

Figure: 32

Injection-6

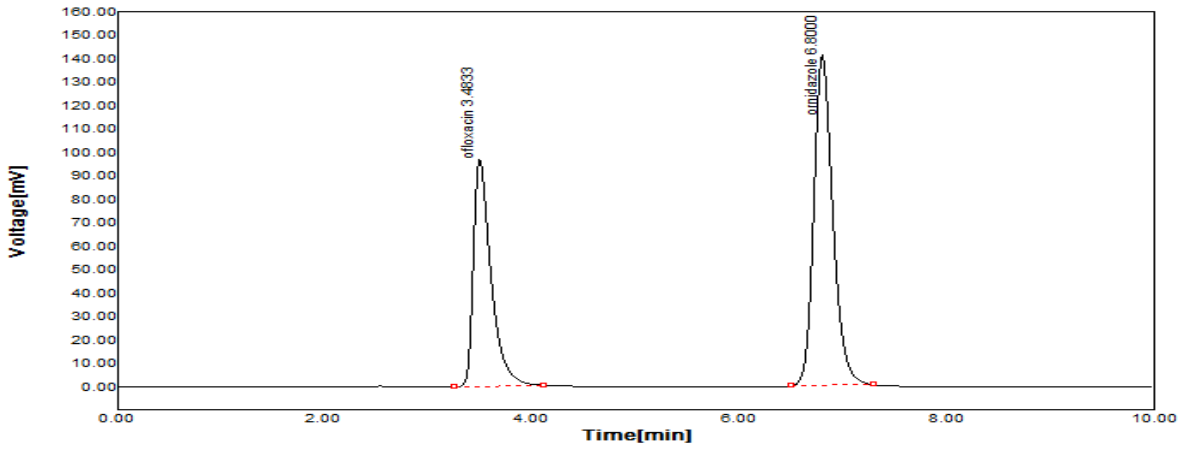


	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.4	1093	2495	1.7
2	ornidazole	6.4	1822	9148	1.2

Figure: 33

Method precision Chromatograms

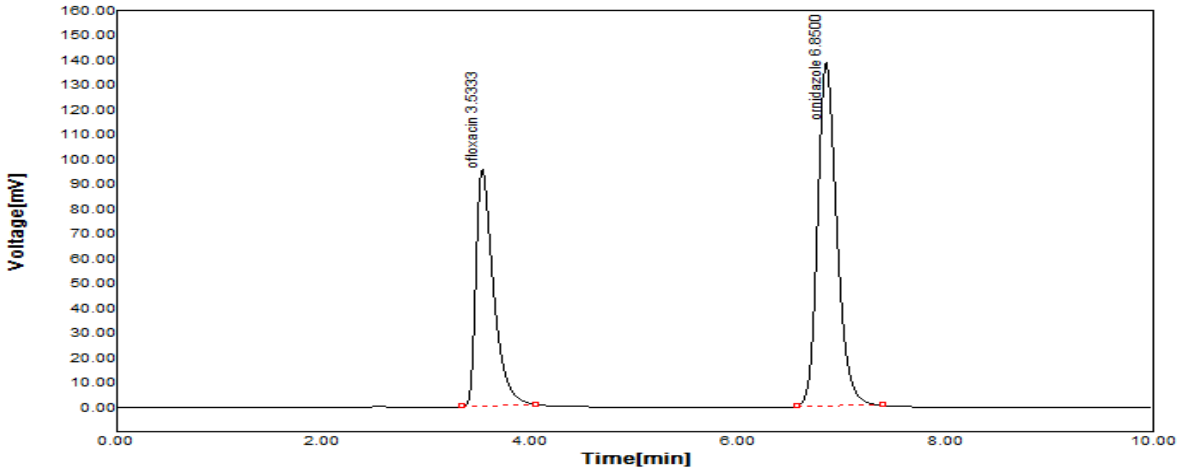
Sample-1:



	Name	RT	Area
1	ofloxacin	3.5	1144
2	ornidazole	6.8	1807

Figure:34

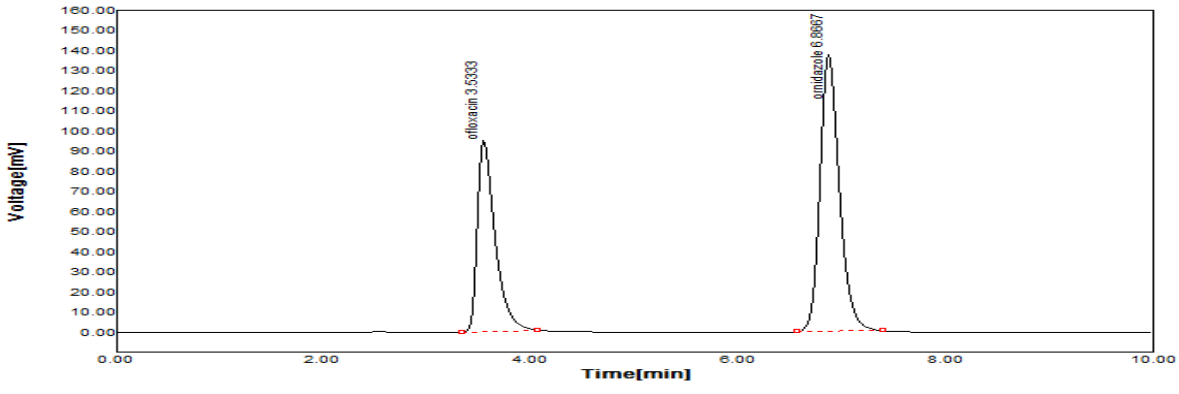
Sample-2:



	Name	RT	Area
1	ofloxacin	3.5	1124
2	ornidazole	6.8	1790

Figure: 35

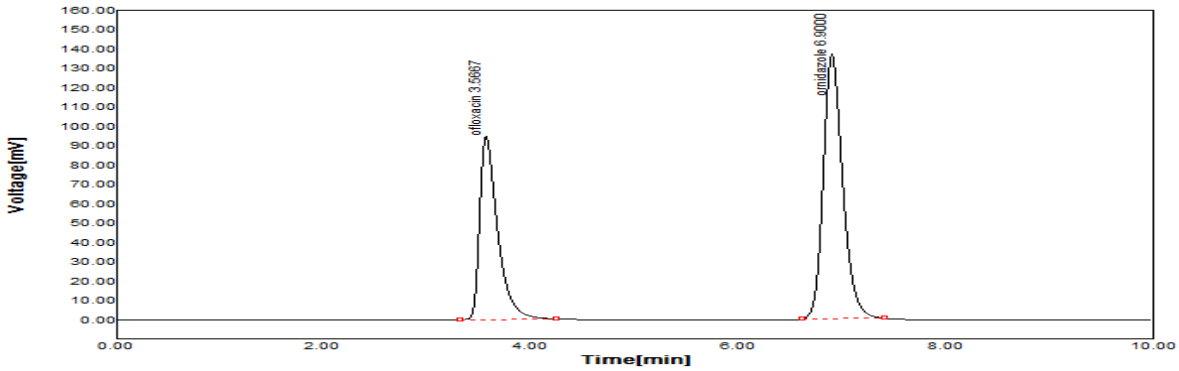
Sample-3:



	Name	RT	Area
1	ofloxacin	3.5	1127
2	ornidazole	6.9	1794

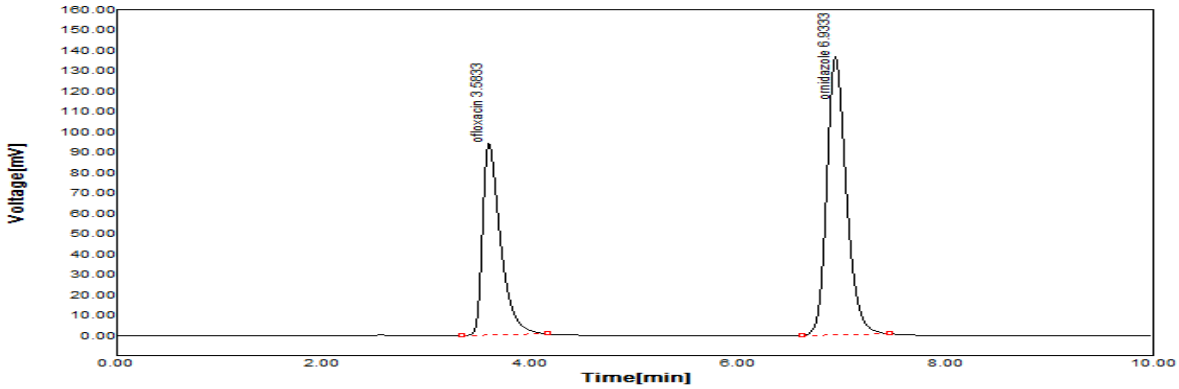
Figure:36

Sample-4:



	Name	RT	Area
1	ofloxacin	3.6	1145
2	ornidazole	6.9	1787

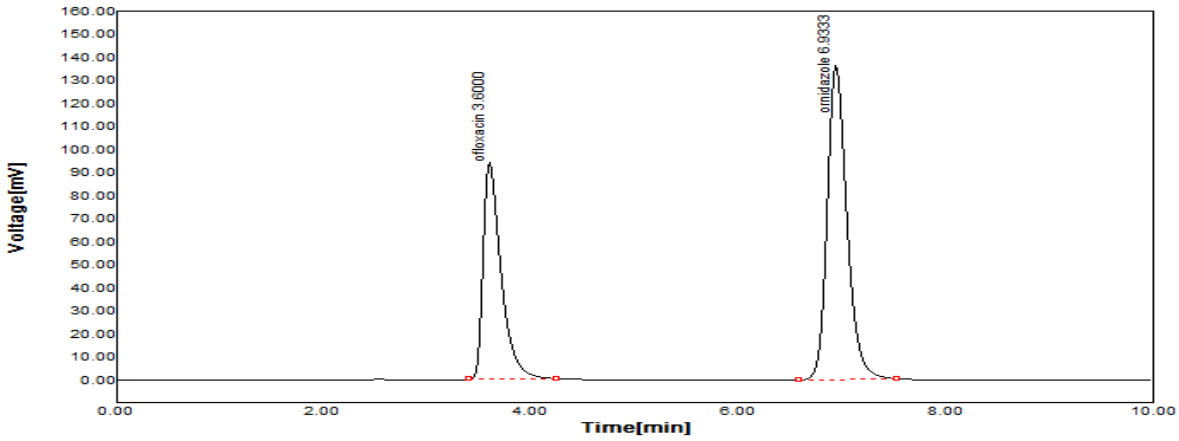
Figure:37



	Name	RT	Area
1	ofloxacin	3.6	1135
2	ornidazole	6.9	1796

Figure: 38

Sample 6:

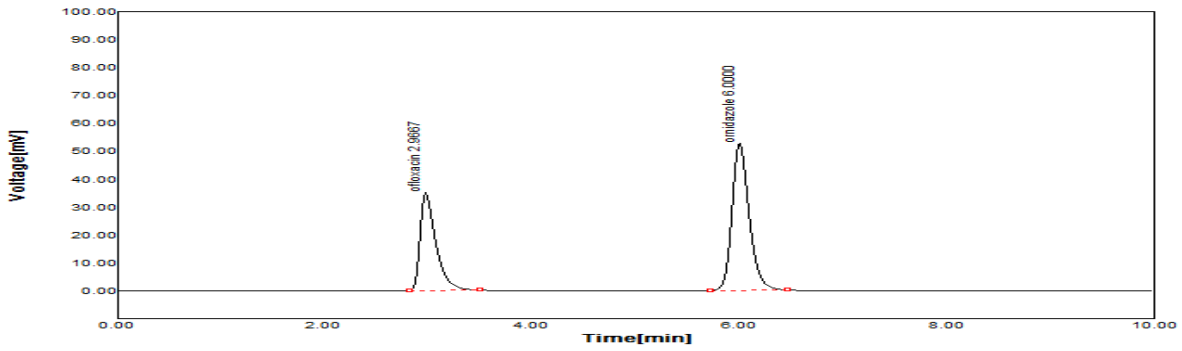


	Name	RT	Area
1	ofloxacin	3.6	1138
2	ornidazole	6.9	1803

Figure: 39

Linearity chromatograms:

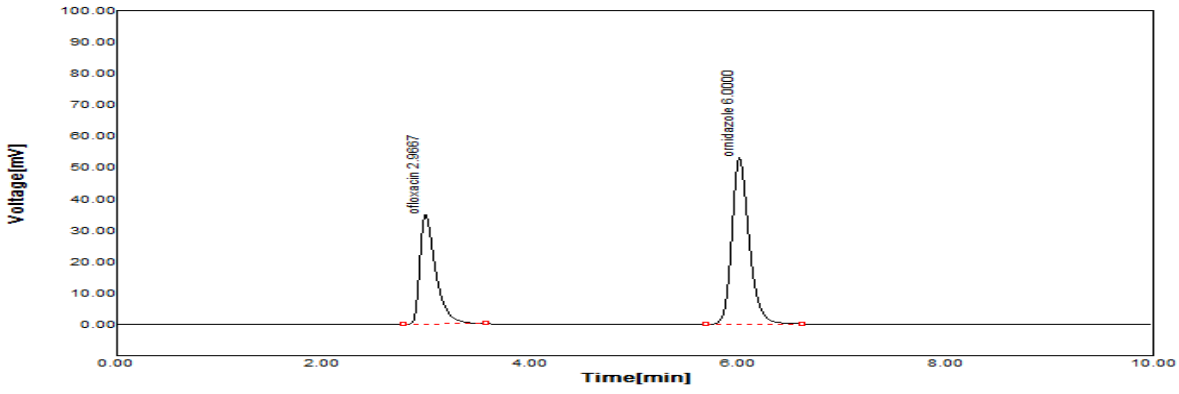
LI-50%-1:



	Name	RT	Area
1	ofloxacin	3.0	370
2	ornidazole	6.0	608

Figure: 40

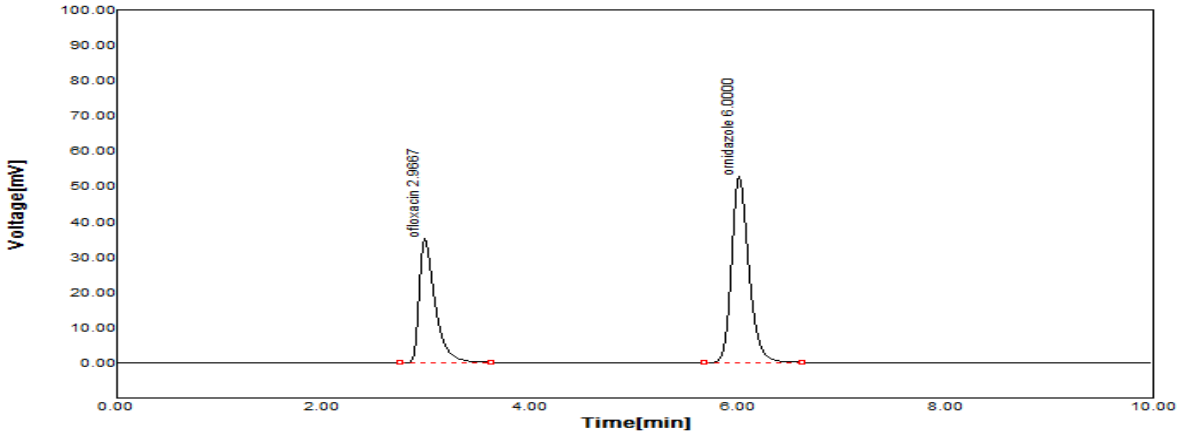
LI-50%-2:



	Name	RT	Area
1	ofloxacin	3.0	373
2	ornidazole	6.0	614

Figure: 41

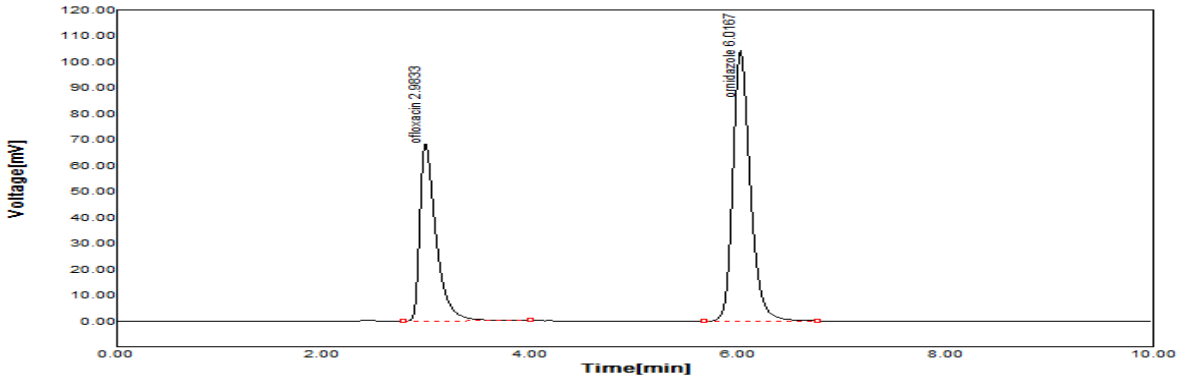
LI-50%-3:



	Name	RT	Area
1	ofloxacin	3.0	374
2	ornidazole	6.0	614

Figure: 42

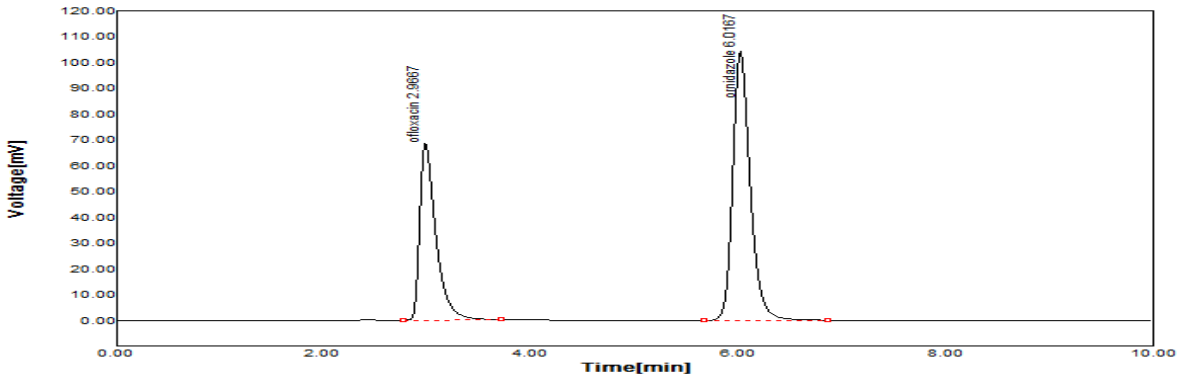
LI-75%-1:



	Name	RT	Area
1	ofloxacin	3.0	746
2	ornidazole	6.0	1218

Figure: 43

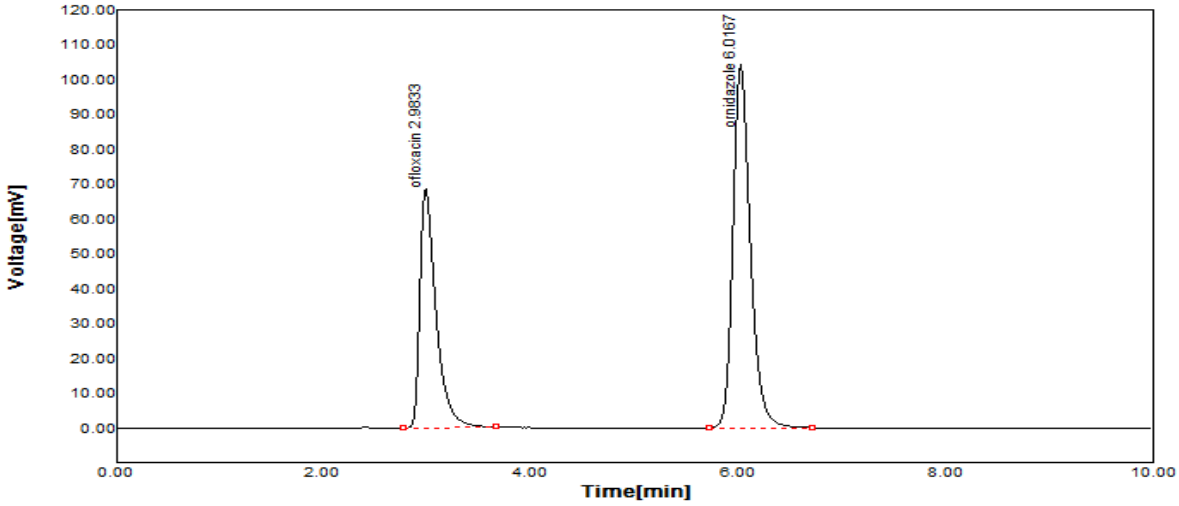
LI-75%-2:



	Name	RT	Area
1	ofloxacin	3.0	743
2	ornidazole	6.0	1224

Figure: 44

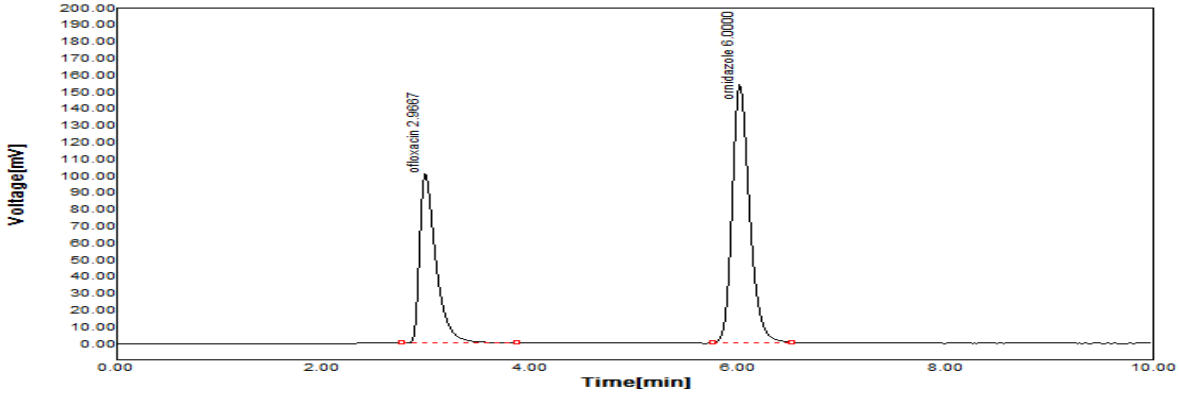
LI-75%-3:



	Name	RT	Area
1	ofloxacin	3.0	742
2	ornidazole	6.0	1219

Figure: 45

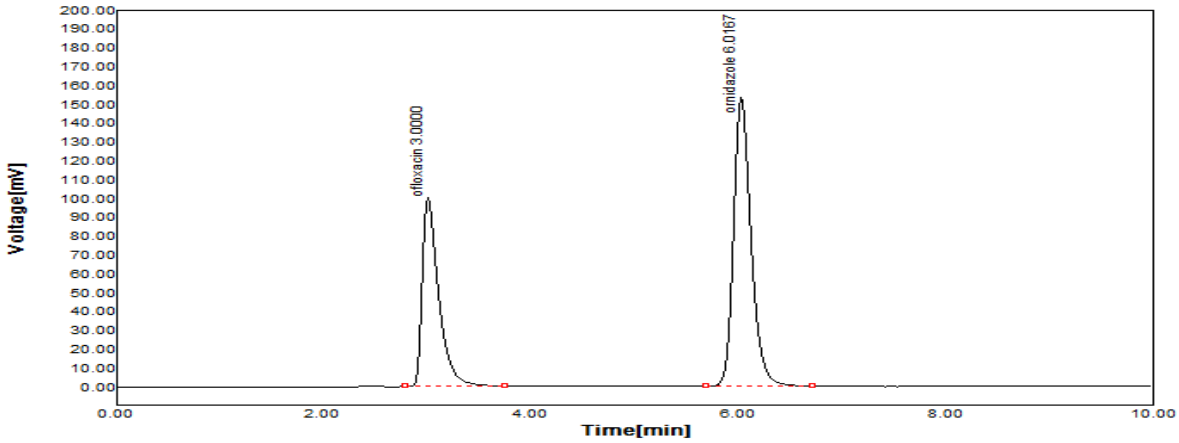
LI-100%-1:



	Name	RT	Area
1	ofloxacin	3.0	1112
2	ornidazole	6.0	1792

Figure: 46

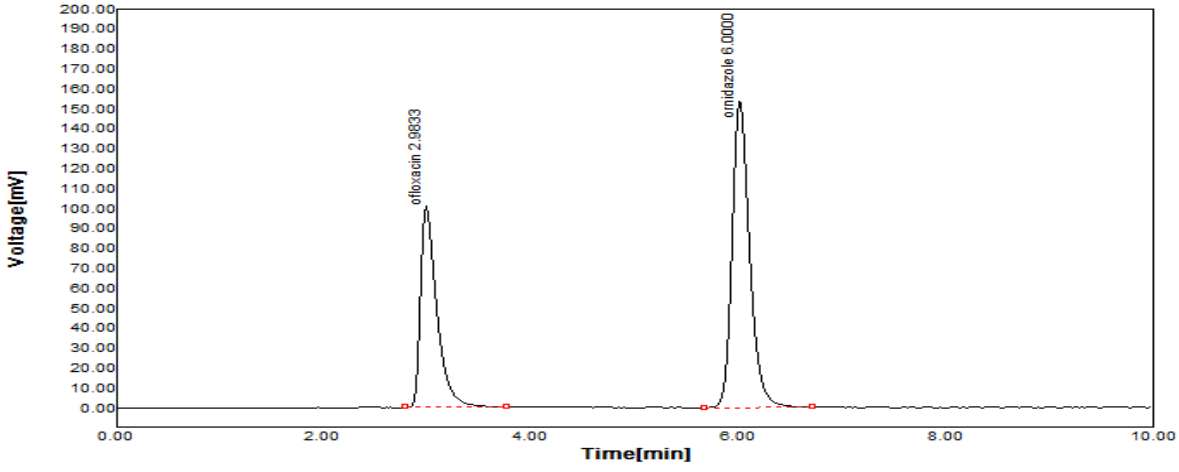
LI-100%-2:



	Name	RT	Area
1	ofloxacin	3.0	1105
2	ornidazole	6.0	1810

Figure: 47

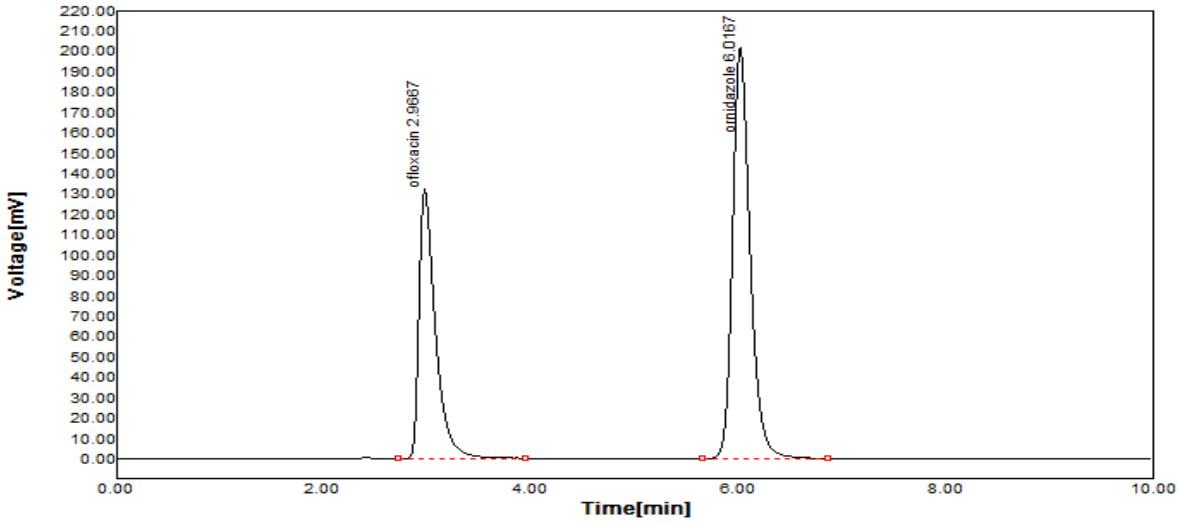
LI-100%-3:



	Name	RT	Area
1	ofloxacin	3.0	1108
2	ornidazole	6.0	1815

Figure: 48

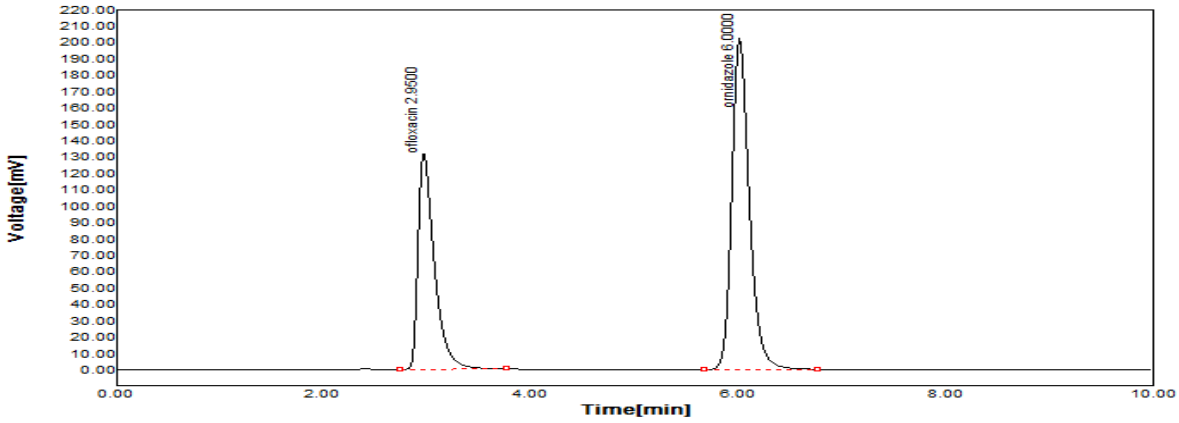
LI-125%-1:



	Name	RT	Area
1	ofloxacin	3.0	1458
2	ornidazole	6.0	2387

Figure: 49

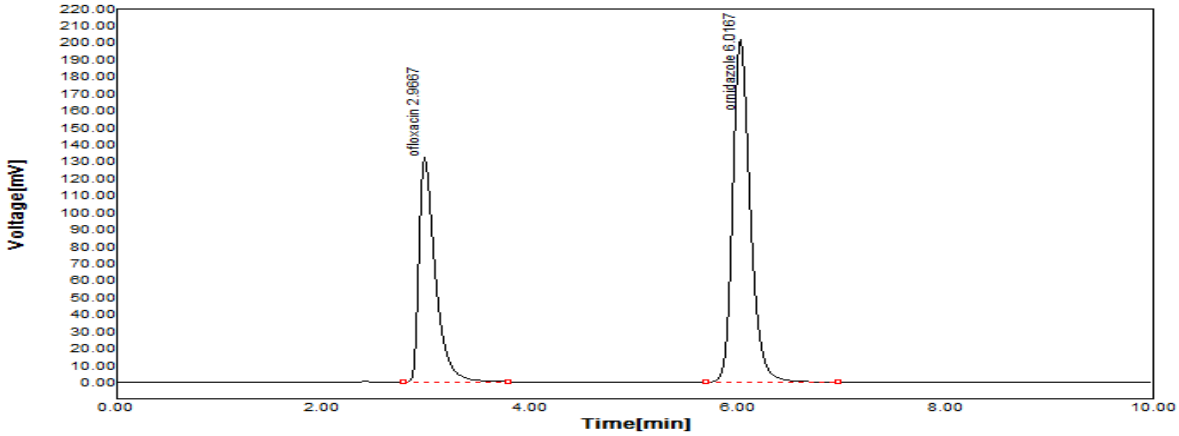
LI-125%-2:



	Name	RT	Area
1	ofloxacin	3.0	1454
2	ornidazole	6.0	2384

Figure:50

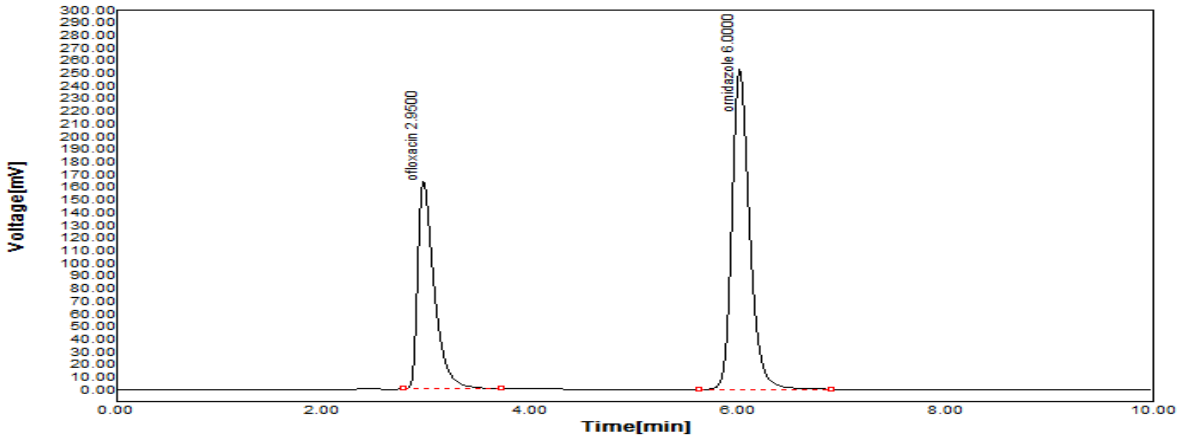
LI-125-3:



	Name	RT	Area
1	ofloxacin	3.0	1455
2	ornidazole	6.0	2392

Figure: 51

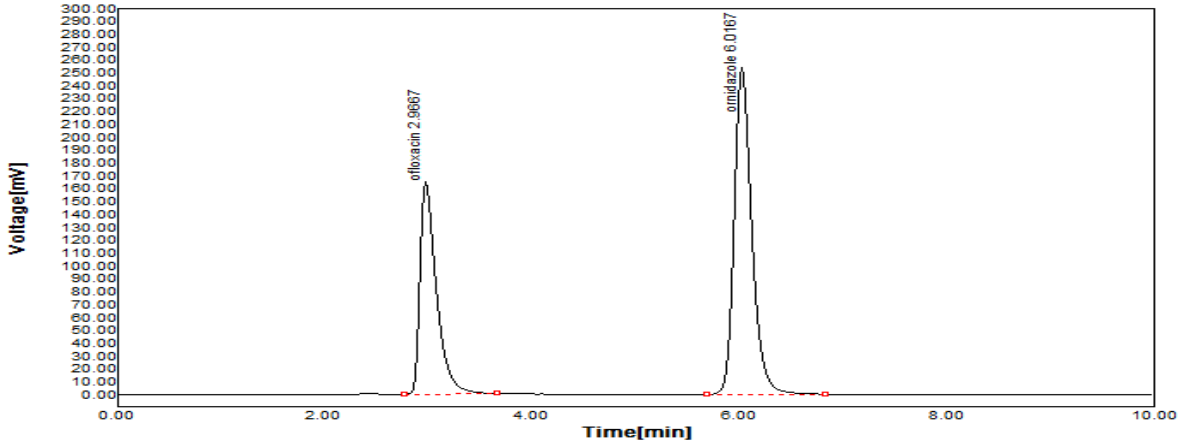
LI-150%-1:



	Name	RT	Area
1	ofloxacin	3.0	1823
2	ornidazole	6.0	3002

Figure: 52

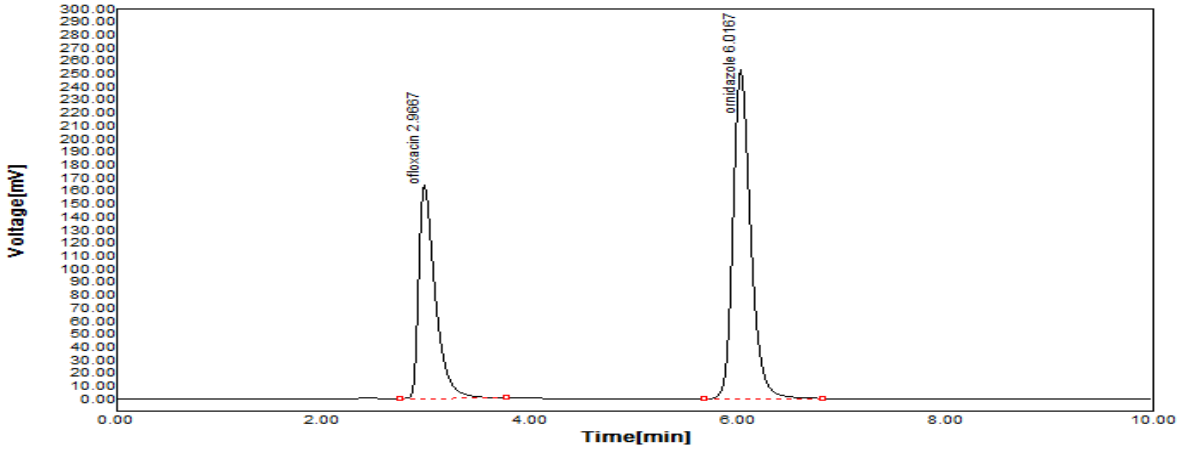
LI-150%-2:



	Name	RT	Area
1	ofloxacin	3.0	1821
2	ornidazole	6.0	2999

Figure: 53

LI-150%-3:

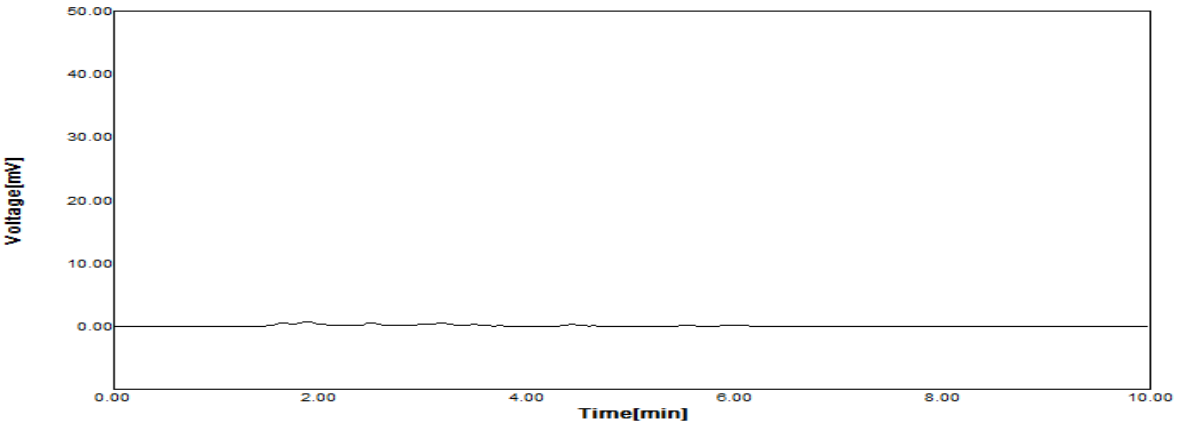


	Name	RT	Area
1	ofloxacin	3.0	1828
2	ornidazole	6.0	2998

Figure: 54

Placebo interference chromatograms

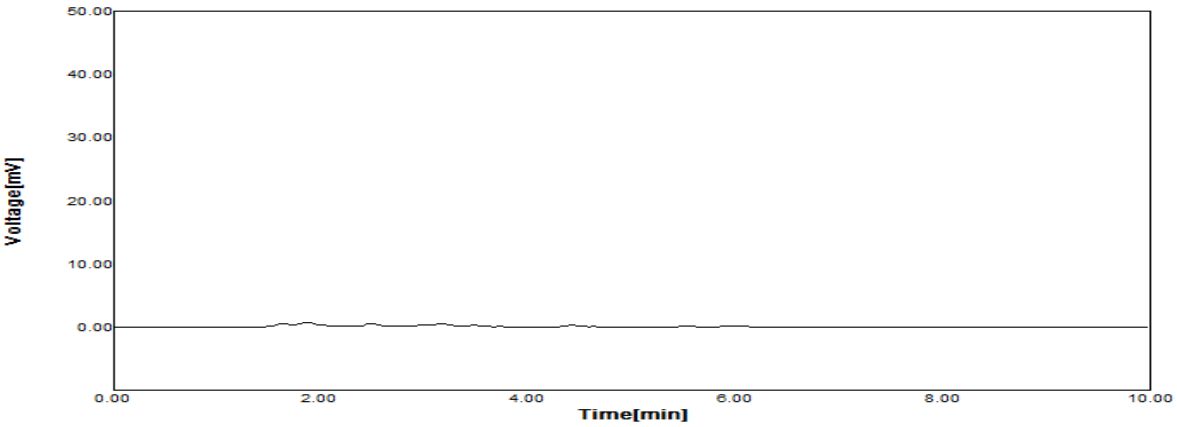
Placebo-1



	Name	RT	Area	%Area
1	ofloxacin	3.0		
2	ornidazole	6.0		

Figure: 55

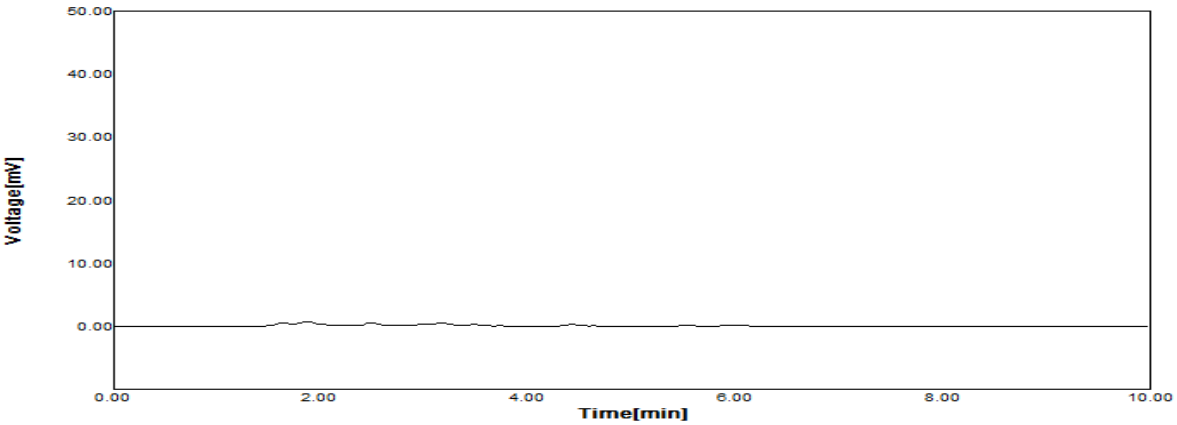
Placebo-2



	Name	RT	Area	%Area
1	ofloxacin	3.0		
2	ornidazole	6.0		

Figure: 56

Placebo-3

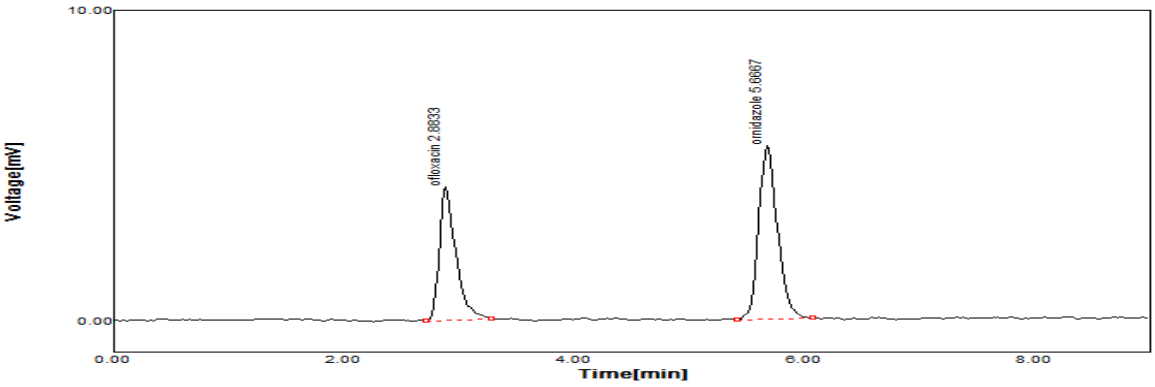


	Name	RT	Area	%Area
1	ofloxacin	3.0		
2	ornidazole	6.0		

Figure: 57

Limit of detection chromatograms:

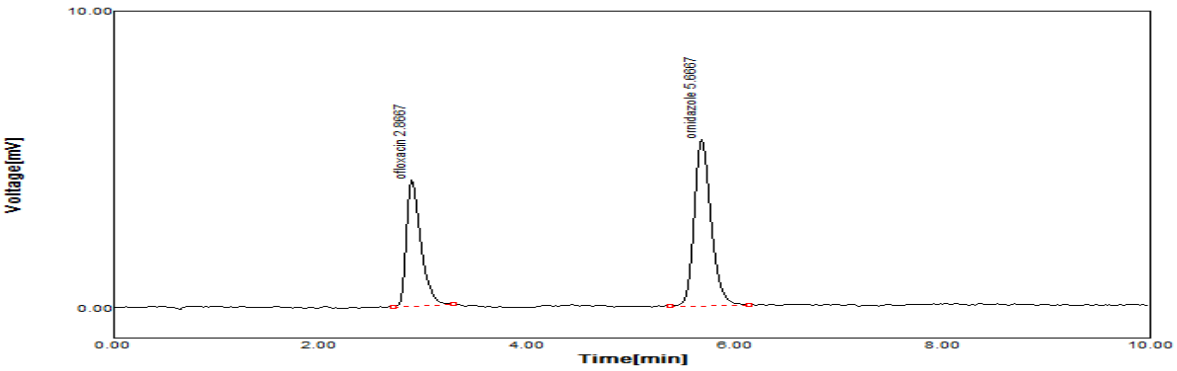
Injection -1



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	2.9	42	2858	1.7
2	ornidazole	5.7	63	8205	1.2

Figure: 58

Injection -2

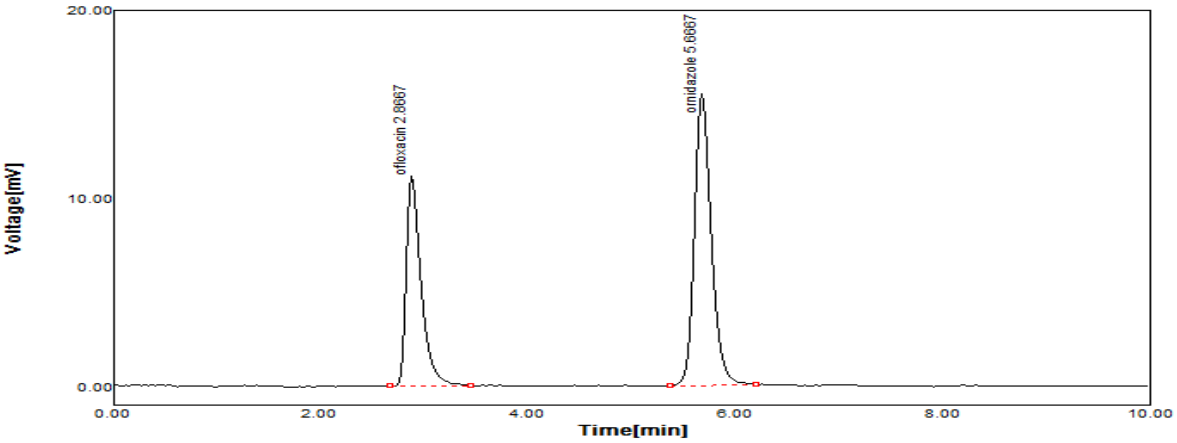


	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	2.9	43	3034	1.6
2	ornidazole	5.7	63	7125	1.3

Figure: 59

Limit of Quantification chromatograms

Injection -1

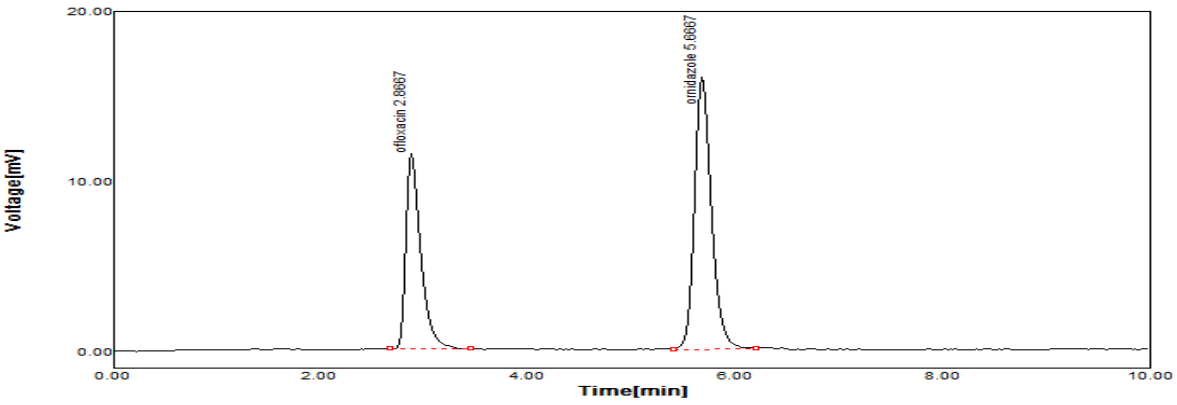


	Name	RT	Area	USP plate count	USP Tailing

1	ofloxacin	2,9	112	2803	1.8
2	ornidazole	5.7	175	7807	1.2

Figure: 60

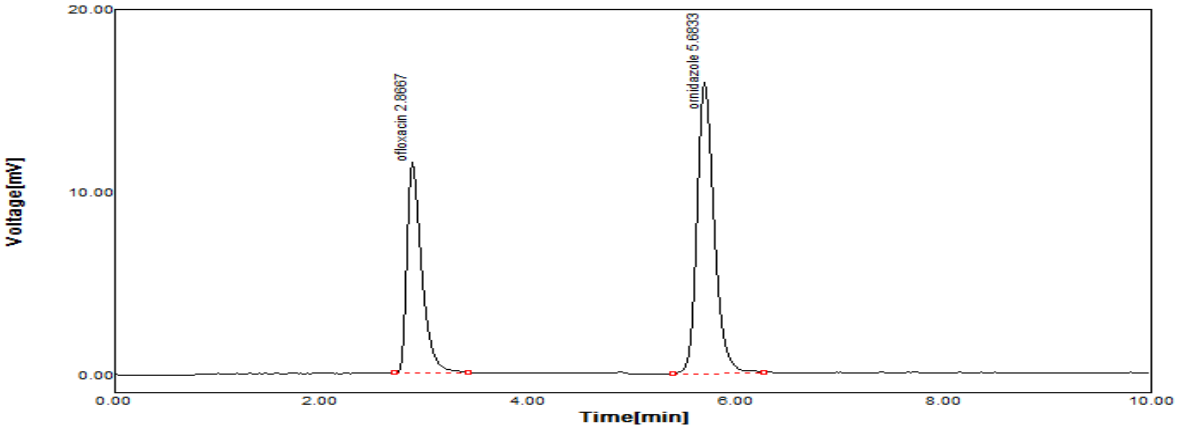
Injection -2



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	2.9	115	2876	1.7
2	ornidazole	5.7	181	7092	1.3

Figure: 61

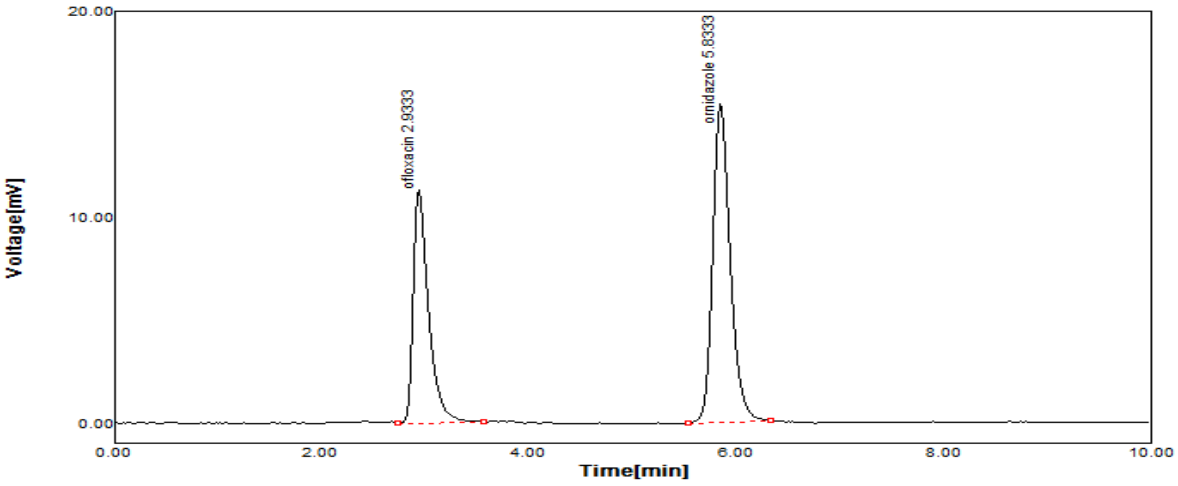
Injection -3



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	2.9	116	2863	1.7
2	ornidazole	5.7	184	6923	1.2

Figure: 62

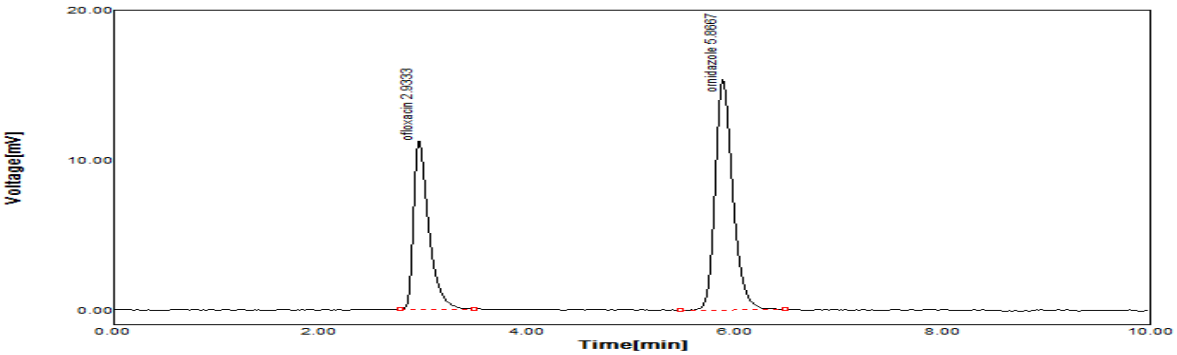
Injection -4



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	2.9	117	2984	1.6
2	ornidazole	5.8	183	6951	1.3

Figure: 63

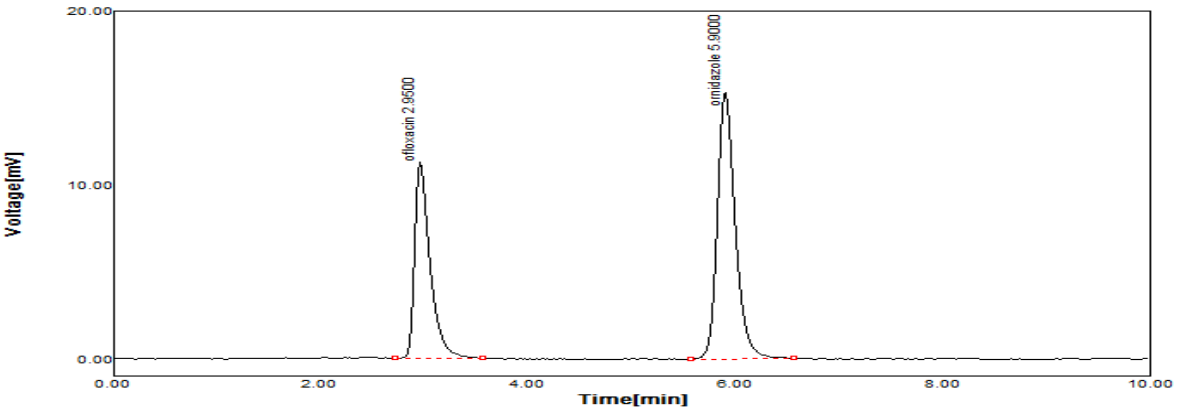
Injection -5



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	2.9	115	2386	1.8
2	ornidazole	5.9	183	6960	1.3

Figure: 64

Injection -6



	Name	RT	Area	USP plate count	USP Tailing
1	ofloxacin	3.0	117	2820	1.8
2	ornidazole	5.9	185	6902	1.2

Figure: 65

RESULT AND DISCUSSION

A simple Reverse phase high performance liquid chromatographic method has been developed subsequently validated for ofloxacin and ornidazole tablets.

The separation was carried out by using a Buffer: Acetonitrile (80:20).The detection was carried at 308nm.The column was Cosmosil C_{18}-MS-II packed column (150 x4.6mm ,5µ).the flow rate selected as 1ml/min.

The retention time of ofloxacin and ornidazole was found to be 3.0 and 6.0. The asymmetry facto tailing factor of ofloxacin and ornidazole was found to be 1.78 and 1.3, which indicates symmetrical na of the peak. The number of theoretical plates of ofloxacin and ornidazole was found to be 2546 6578,which indicates the efficient Performance of the column .These parameters represent the specificit the method.

From the linearity studies, specified concentration levels were determined. it was observed ofloxacin and ornidazole were linear in the range of 50% to 150% for the target concentration by HPLC.The linearity range of ofloxacin and ornidazole 50% to 150% was found to obey linearity wi correlation coefficient to 0.9998,and 0.9999. The validation of the proposed method was verified system precision and method precision by RP-HPLC.The %RSD of system suitability for ofloxacin ornidazole was found to be 0.24,0.49.

The validation of the proposed method was verified by recovery studies. The percentage recov range was found to be satisfied which represent in results. The robustness studieswere performed changing the flow rate , filters and wavelength.The ruggedness study was also

performed.The analytical method validation was carried out by RP-HPLC as per ICH guidelines and gi below are the tables are the summary of the results.

Analytical method validation report for ofloxacin and ornidazole tablets

Table -24

S.No	Parameter	Experiment	Observation	Acceptance criteria
01	System suitability	System suitability Ofloxacin	0.24%	RSD NMT 2.0%
			1.78	Tailing factor NMT 2.0
		System suitability Ornidazole	0.49%	RSD NMT 2.0%
			1.3	Tailing factor NMT 2.0
02	Specificity	Placebo interference	Placebo not shown any peak at the retention time of ofloxacin and ornidazole peak.	Placebo should not show any peak at the retention time of ofloxacin and ornidazole peak
03	Limit of detection	Ofloxacin	0.001mg/ml	NMT 2
		Ornidazole	0.0025mg/ml	NMT 2
	Limit of quantitation	Ofloxacin	0.003 mg/ml	NMT 10
		Ornidazole	0.0075mg/ml	NMT 10

S.No	Parameter	Experiment	Observation		Acceptance criteria
04	Linearity	Correlation coefficient for ofloxacin	0.9998		NLT 0.999
		Correlation coefficient for Ornidazole	0.9999		NLT 0.999
05	Precision	Repeatability for ofloxacin	0.60%		%RSD NMT 2.0%
		Repeatability for ornidazole	1.02%		%RSD NMT 2.0%
		Method precision For ofloxacin	0.21%		%RSD NMT 2.0%
			100.7%		Assay: 90 .0% to 110.0%
		Method precision For ornidazole	0.16%		%RSD NMT 2.0%
			99.6%		Assay:90 .0% to 110.0%
06	Range	Accuracy for ofloxacin	Spike level	%Mean recovery	Mean recovery should be within 98.0% to 102.0%
			50%	99.1%	
			100%	98.9%	
			150%	99.1%	
		Accuracy for ornidazole	50%	98.7%	
			100%	98.5%	
			150%	98.8%	

S.No	Parameter	Experiment	Observation	Acceptance criteria
07	Ruggedness	Analyst-1 Ofloxacin :0.60% Ornidazole:1.02%	Analyst-II Ofloxacin :0.24% Ornidazole:0.49%	%RSD NMT 2.0%
08	Robustness	Effect of variation in flow rate	System suitability Pass	System suitability should pass
			Allowable variation : 0.9 to 1.1 ml/min	
		Effect of variation in composition of mobile phase	System suitability Pass	System suitability should pass
			Allowable variation :90% to 110% of the method highest organic phase	
		Filter validation	This is no effect of filtered and centrifuged sample	Similarity factor of centrifuged against filtered should be in 0.98% to 1.02%

CONCLUSION

A RP-HPLC method for ofolaxacin and ornidazole were developed and validated in tablet dosage form as per ICH guidelines. The results are found to be complying with the acceptance criteria for each of the parameter.

Shimadzu HPLC(Autochrome 3000 software with UV detector) with Cosmosil C_{18} –MS –II (150X 4.6MM, 5µ) Packed Column, Injection volume of 20µl is injected and eluted with the Mobile phase (Buffer and CAN, in the ratio of 80:20) Which was pumped at a flow rate of 1.0 ml at 308nm.The peak of ofloxacin and ornidazole was found well separated at 3.0min , 6.0min.The developed method was validated for various parameters as per ICH guidelines like system suitability,accuracy,precision, linearity , specificity, limit of detection ,limit of quantitation ,ruggedness,robustness.

Hence it is concluded that the assay method is found to be valid in terms of reliability, precision, accuracy and specificity and hence it is suitable for routine anaclysis as well as for stability analysis.

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