METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF CEFUROXIME AXETIL AND LINEZOLID IN PHARMACEUTICAL DOSAGE FORM BY RP-HPLC AND HPTLC METHOD

Dissertation work submitted to The Tamilnadu Dr. M.G.R Medical University, Chennai in partial fulfilment for the award of degree ***of***

MASTER OF PHARMACY

IN

Pharmaceutical Analysis

Submitted by

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This is to certify that **Mr.PRASANTH .A .R**. who is studying second year M.Pharm Analysis in RVS college of pharmacy has done his project in our Laboratory Care Keralam,Koratty,Thrissur,Kerala with specification of Development and validation for the simultaneous estimation of Cefuroxime axetil and Linezolid in Pharmaceutical dosage form by RP-HPLC and HPTLC Method from 04.05.2016

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Dr.W.D.SAM SOLOMON,M.Pharm,PhD

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Evaluation Certificate

Dissertation title : "Method Development and validation for the simultaneous estimation of Cefuroxime axetil and Linezolid in pharmaceutical dosage form by RP-HPLC and HPTLC"

Name of the Candidate : Mr.PRASANTH A R

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INTERNAL EXAMINER **EXTERNAL EXAMINER**

Date: Date:

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PRASANTH A R

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CONTENTS

1. INTRODUCTION

1.1. ANALYTICAL CHEMISTRY [1-4]

Analytical Chemistry is defined as “The science and the art of determining the composition of materials in terms of the elements or compounds contained.” This branch of chemistry, which deals with both theoretical, practical science and practiced in a large number of laboratories in many diverse ways. Methods of analysis are routinely developed, improved, validated, collaboratively studied and applied. In analytical chemistry it is of prime importance to gain information about the qualitative and quantitative composition of substances and chemical species that is to find out what substance is composed and exactly how much. In quantitative analysis the question is how much is present. The research work in this thesis is based on this criterion. Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations. Quality is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods there can be no “second quality” in drugs. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production.

1.2. IMPORTANCE OF DRUG ANALYSIS

Medicines are key part of the health care system. Numerous medicines are introduced into the world-market and also, that is increasing every year.These medicines are being either new entities or partial structural modification of the existing one. So, evaluation of quality and efficacy of these medicines are important Right from the beginning of discovery of any medicine, quality and efficacy of the same are checked by quantification means. Quality and efficacy are checked by either observing effect of drug on various animal models or by analytical means the option of animal models is not practically suitable for every batch of medicine as it requires long time, high cost and more man-power. Later option of analytical way is more suitable, highly precise, safe and selective.

The analytical way deals with quality standards which are assigned for products to have desirable efficacy of the medicines. Sample representing any batch are analyzed for these standards and it is assumed that drug/medicine complying with those standards are having desired effect on use. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stage of production. The decision to release or reject a product is based on one or more type of control action. Due to rapid growth of pharmaceutical industry during last several years, number of pharmaceutical formulations enter as a part of health care system and thus, there has been rapid progress in the field of pharmaceutical analysis. Developing analytical method for newly introduced pharmaceutical formulation is a matter of most importance because drug or drug combination may not be official in any pharmacopoeias and thus, no analytical method for quantification is available. To check the quality standards of the medicine various analytical methods are used. Modern analytical techniques are playing key role in assessing chemical quality standards of medicine. Thus analytical techniques are required for fixing standards of medicines and its regular checking. Out of all analytical techniques, the technique which is widely used to check the quality of drug is known as "chromatography'.

1.3. Relevance of Analytical Methods [5-7]

Analytical methods which are a measure of quality of the drugs play a very comprehensive role in drug development and follow up activities. It assures that a drug product meets the established standard, is stable and will continue to meet purported quality throughout its shelf life.These methods should be selective and sensitive to monitor the known and unknown impurities and have to be written in a format such that they can be reproduced over a period of time and from laboratory to laboratory, i.e., these methods should be validated.

1.4. Analytical methods

Pharmaceutical analysis plays an important role right from the testing of raw materials; in-process quality checks and analysis of finished products.

Pharmaceutical analysis is considered to determine the identity, strength, quality and purity of drug samples.

In analytical chemistry, it is of prime importance to gain information about the qualitative and quantitative compositions of substances and chemical species, that is, to find out what a substance is composed of and exactly how much. In general terms, pharmaceutical analysis comprises of those procedures necessary to determine the "identity, strength, quality and purity" of drugs.

Analytical methods are required to characterize the drug substances and drug product composition during all phases of pharmaceutical development. Early phase methods must support changes in synthetic routes and dosage form and elucidate the structures and levels of impurities. In later phases, goals change to the development of rapid and robust methods for release and stability evaluation.

Analysis includes a wide range of simple and instrumental analytical methods, but the most widely used methods for quality assurance are spectroscopy and chromatography. Most quantitative analysis requires measuring specified components in the presence of sample matrix and/or related substances; therefore isolation or separation of the components are required preceding such analysis. In such cases chromatographic techniques are used for quantitative analysis. In case, where matrix interference is not observed quantitative measurements are made using spectroscopic or titration methods.

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

1.5. High Performance Liquid Chromatography

The term *chromatography* meaning "color writing," was first discovered by Mikhail Tswett, a Russian botanist who separated plant pigments on chalk ($CaCO_3$) packed in glass columns in 1903. High pressure liquid

chromatography was developed in the mid-1970's andquickly improved with the development of column packing materials and the additional convenience of online detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds. By the 1980's HPLC was commonly used for the separation of chemical compounds. Computers and automation added to the convenience of HPLC.

Liquid chromatography (LC) is a physical separation technique conducted in the liquid phase. Analyte is forced to flow through a column under high pressure. Then it is separated into its constituent components by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column).

Four major separation modes of HPLC are normal phase, reversed phase, ion exchange chromatography, and size exclusion chromatography (gel permeation and gel filtration chromatography.

Normal-Phase Chromatography (NPC)[8-9]

NPC is the traditional separation mode based on adsorption/desorption of the analyte onto a polar stationary phase (typically silica or alumina). In this technique, nonpolar compounds travel faster and are eluted first because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for a longer time because of their higher affinity towards the stationary phase. Normal phase mode of separation is, therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

Reversed-Phase Chromatography (RPC)

Reversed phase mode is the most popular mode for analytical and preparative separations of compounds of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octadecyl functional group bonded to silica gel and the mobile phase is a polar solvent. An aqueous mobile phase

allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster.The different columns used are octadecylsilane (ODS) or C_{18}, C_8, C_4 etc., (in the order of increasing polarity of the stationary phase).

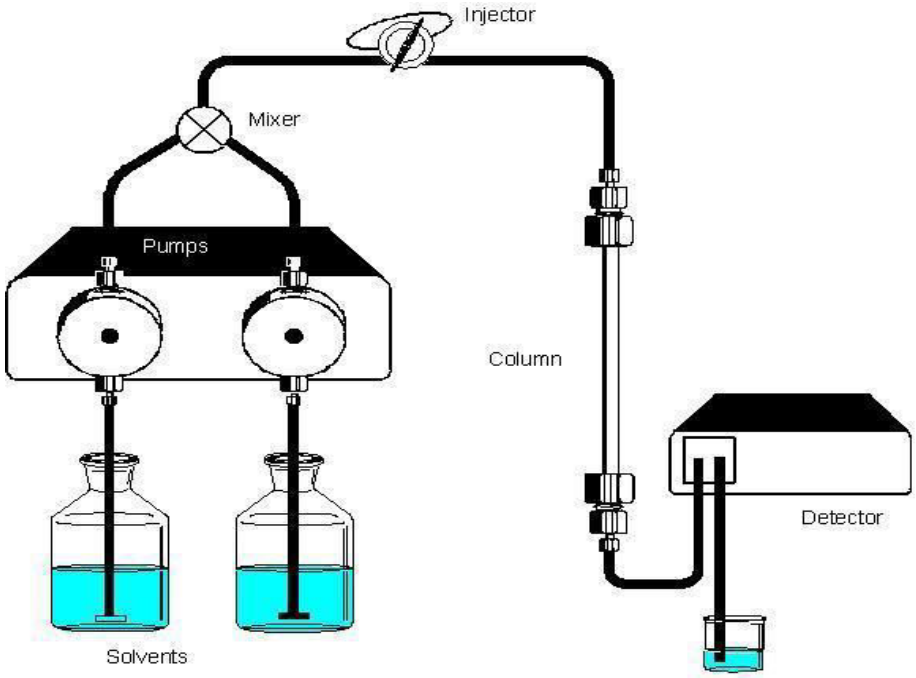


Fig 1: Principle pattern of a HPLC instrument

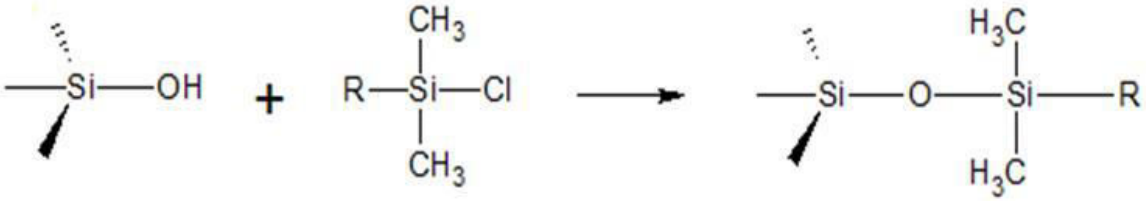
Columns are the heart of HPLC. Liquid chromatographic columns are usually constructed from smooth bore stainless steel tubing. Sometimes made from heavy walled glass tubings and polymer tubings such as PEEK. Guard columns are introduced before analytical columns to increase the life of analytical columns, by removing not only particulate matter and contaminants from solvents but also sample components that bind irreversibly to the stationary phase. Analytical columns ranges from 5 - 25 cm long; inside diameter is often 3 - 5 mm; the most common particle size of packing is 3 - 5 µm

Two basic types of column packing used in LC are pellicular and porous particles. In pellicular packing, spherical, nonporous, glass or polymer beads are

used. A thin layer of silica, alumina, polystyrene – divinylbenzene synthetic resin, or an ion – exchange resin was deposited on the surface of these beads. In the typical porous particle packing of LC is composed of silica, alumina, polystyrene – divinylbenzene synthetic resin, or an ion – exchange resin.

Columns for the bonded phase chromatography is prepared by surface functionalization of silica. The surface of fully hydrolysed silica is made up of chemically reactive silanol groups.

The most useful bonded phase coatings are siloxanes formed by the reaction of the hydrolyzed surface with organochlorosilanes. For example,



Reaction of silanol group with organochlorosilane leads to formation of siloxanes

Where R is an alkyl group or substituted alkyl group like C_8, C_{18} Different types of detectors used in HPLC are absorbance detectors, fluorescence detectors, electrochemical detectors, refractive index detectors, conductivity detectors, photo ionization detectors etc.

Method Development and Design of Separation Method[10-13]

Methods for analysing drugs in single or multi component dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase

chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble.

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 5-10 % organic phase in the mobile phase and the organic phase concentration (methanol or acetonitrile) can be increased up to 100 % within 30-45 min. Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted,namely at what mobile phase composition.

Changing the polarity of mobile phase can alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by the proper selection of pH. The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase of the organic phase concentration is needed.

In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual present in the sample. If analyte standards are available, their UV spectra can be measured prior to HPLC method development.

The molar absorbance at the detection wavelength is also an important parameter. When peaks are not detected in the chromatograms, it is possible that the sample quantity is not enough for the detection. An injection of volume of 20 µL from a solution of 1 mg/mL concentration normally provides good signals for UV active compounds around 220 nm. Even if the compounds exhibit higher λ_{max}, they absorb strongly at lower wavelength.

It is not always necessary to detect compounds at their maximum absorbance. It is, however, advantageous to avoid the detection at the sloppy part of the UV spectrum for precise quantitation. When acceptable peaks are detected on the chromatogram, the investigation of the peak shapes can help further method development.

The addition of peak modifiers to the mobile phase can affect the separation of ionic samples. For examples, the retention of the basic compounds can be influenced by the addition of small amounts of triethylamine (a peak modifier) to the mobile phase. Similarly for acidic compounds small amounts of acids such as acetic acid can be used. This can lead to useful changes in selectivity. When tailing or fronting is observed, it means that the mobile phase is not totally compatible with the solutes. In most case the pH is not properly selected and hence partial dissociation or protonation takes place. When the peak shape does not improve by lower (1-2) or higher (8-9) pH, then ion-pair chromatography can be used. For acidic compounds, cationic ion pair molecules at higher pH and for basic compounds, anionic ion-pair molecules at lower pH can be used. For amphoteric solutes or a mixture of acidic and basic compounds, ion-pair chromatography is the method of choice.

The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvents for the preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column or injector.

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks on the chromatogram detect all the compounds. By sight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less runtime.

The peak resolution can be increased by using a more efficient column (column with higher theoretical plate number, N) which can be achieved by using a column of smaller particle size, or a longer column. These factors, however, will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.

Unfortunately, theoretical predictions of mobile phase and stationary phase interactions with a given set of sample components are not always accurate, but they do help to narrow down the choices for method development. The separation scientist must usually perform a series of trial and error experiments with different mobile phase compositions until a satisfactory separation is achieved.

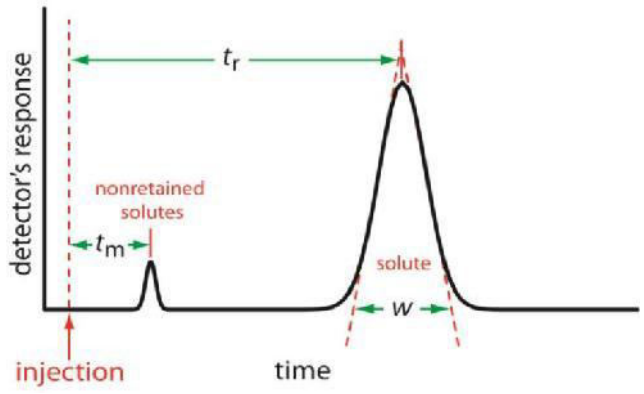


Fig 2: A HPLC chromatogram

The parameters that are affected by the changes in chromatographic conditions are:

1. Resolution (RS).
2. Capacity factor (k‟).
3. Selectivity (α).
4. Plate number (N).
5. Asymmetry factor (T).

Quantitative Analysis in HPLC[14]

Two methods are generally used for quantitative analysis. They are the external standard method, the internal standard method and the standard addition method.

1. External standard method

The external standard method involves the use of a single standard or up to three standard solutions. The peak area or the height of the sample and the standard used are compared directly or the slope of the calibration curve based on standards that contain known concentrations of the compounds of interest.

2. Internal standard method

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms, to compensate for the losses of the compounds of interest during sample pretreatment steps. Any loss of the component of interest will be accompanied by the loss of an equivalent fraction of internal standard. The accuracy of this approach obviously dependents on the structural equivalence of the compounds of interest and the internal standard.

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it. Response factor is used to determine the concentration of a sample component in the original sample. The response factor (RF) is the ratio of peak areas of sample component (A_X) and the internal standard (A_{ISTD}) obtained by injecting the same quantity.

1.6. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY[15-19]

The basic principle of Thin Layer Chromatography is adsorption. The major components of TLC are a stationary phase and a mobile phase. The stationary phase is a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent usually silica gel, aluminium oxide, or cellulose. After the

application of the sample on the stationary phase, the mobile phase is allowed to move through the stationary phase via capillary action. The separation of the components in the sample takes place depended on the affinity of the components towards the stationary phase and mobile phase. Organic solvent or mixture of solvents are used as mobile phase to achieve a good resolution.

High performance thin layer chromatography (HPTLC) is a sophisticated instrumental technique based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation, etc. enable it to be a powerful analytical tool for chromatographic information of complex mixtures of inorganic, organic, and biomolecules. The term HPTLC is used for the technique in which substances are accurately and precisely assayed using high performance grades of silica gel. In HPTLC, the sorbent material like silica gel G60 has finer particle size distribution than conventional TLC material. It is a powerful, reliable and cost effective method for qualitative and quantitative analysis. In HPTLC, the mobile phase moves through the pre-coated stationary phase by capillary action or by gravity.

The position of any solute spot in TLC is characterized by its retention/retardation factor Rf. It is

a fundamental qualitative value and is expressed

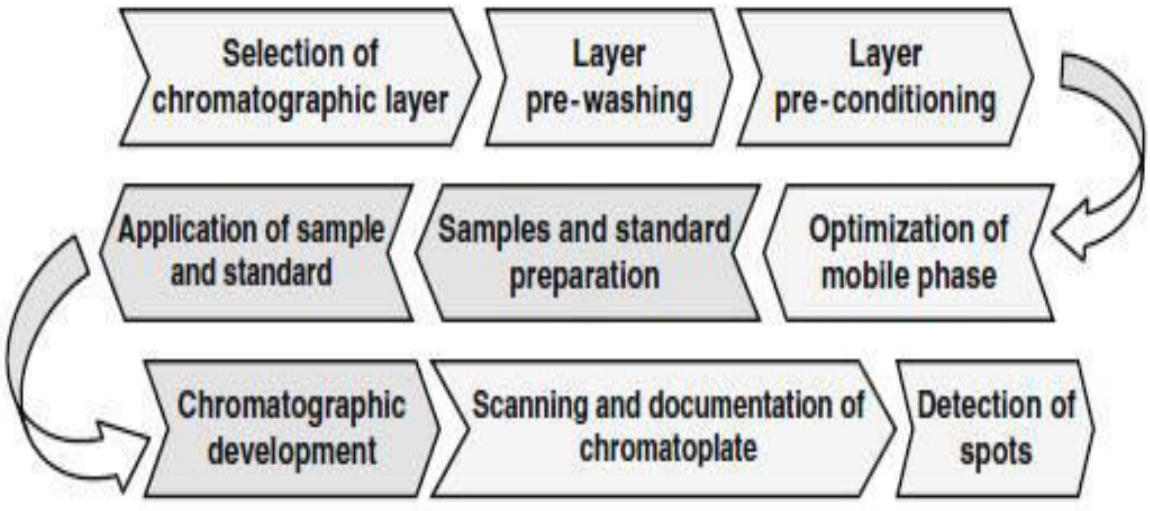
$$Rf = \frac{\text{Distance travelled by solute from application line}}{\text{Distance travelled by solvent from application line}}$$

Rf values range from 1.0 for analyte migrating to the solvent front to 0.0 for an analyte strongly retained at the point of application. The reproducibility of Rf values depends on many factors, such as quality of the sorbent, humidity, layer thickness, development distance, and ambient temperature. Overloading of sample usually results in a slight increase in Rf value.

Features of HPTLC:

1. Simultaneous processing of sample and standard under the same conditions leads to better analytical precision and accuracy.
2. High sample throughput of similar or different nature of samples.
3. Simple sample preparation- handles samples of divergent nature.
4. No prior treatment for solvents like filtration and degassing.
5. Low mobile phase consumption per sample.
6. No interference from previous analysis - fresh stationary and mobile phases - for each analysis – no contamination.
7. Entire spectrum can be seen at a glance.
8. Lower analysis time and less cost per analysis.
9. Low maintenance cost.

Steps involved in HPTLC



Steps involved in the method development of HPTLC

1. Selection of chromatographic layer:

- Pre-coated plates with different support materials like glass, aluminium and plastic and with different sorbent layers are available. Commonly used pre-coated plates are silica gel G 60F_{254}, aluminium oxide, cellulose, hybrid plates etc., plates in size of 10x10cm are usually used.
- 80 % of analysis: Basic substances, alkaloids and steroids - Silica gel G 60F_{254}.
- Amino acids, dipeptides, sugars and alkaloids – cellulose
- Non polar substances, fatty acids, carotenoids, cholesterol – RP-2, RP-8 and RP-18.

2. Sample and standard preparation:

- Proper sample and standard preparation is an important pre-requisite for the success of HPTLC. The choice of suitable solvent for a given analysis is very important. For normal phase mode, non polar solvent should be used for dissolving the sample and standard. For reverse phase mode, polar solvent should be used for dissolving sample and standard.

3. Activation of pre-coated plates:

- Freshly open box of plates do not require activation, plates exposed to high humidity or kept on hand for long time to be activated, by placing in an oven at 110-120º C for 30 minutes prior to spotting,
- Aluminum sheets should be kept in between two glass plates and placing in oven at 110-120º C for 15 minutes.

4. Application of sample and standard:

- The selection of the sample application technique and device to be used depends on: Nature of the analytical work, sample volume, number of samples to be applied, qualitative or quantitative work.
- Sample application should be done either as spots or as bands.
- For sample application as bands, mechanized spotting device called Linomat is used.

5. Selection of mobile phase:

- The mobile phase should be chosen taking into consideration the chemical properties of the analyte and adsorbent.
- Trial and error.
- One"s own experience and Literature.
- Composition is expressed by volume (v/v) and sum of volumes is usually 100.
- **Normal phase:** Mobile phase is non-polar. Non-polar compounds eluted first because of lower affinity with stationary phase. Polar compounds retained because of higher affinity with the stationary phase. Stationary phase is polar.
- **Reverse phase:** Mobile phase is polar. Polar compounds eluted first because of lower affinity with stationary phase. Non-Polar compounds retained because of higher affinity with the stationary phase. Stationary phase is non polar.

6. Pre-conditioning (Chamber saturation):

Unsaturated chamber causes high R_f values.Saturated chamber by lining with filter paper for 30 minutes prior to development- uniform distribution of solvent vapours- less solvent for the sample to travel lower Rf values.

7. Chromatographic development and drying:

- Ascending, descending and two dimensional are the most common methods of development. After the development, the plate is removed from the chamber and dry. Twin trough chambers with stainless steel lid are used for the development of chromatogram.

8. Detection and visualization:

- Detection under UV light is first choice as it is nondestructive, spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length), spots of nonfluorescent compounds like Ethambutol, Dicylomine etc-dipping the plates in0.1 % iodine solution. When individual component does not respond to UV- derivatization required for detection.

9. Quantification:

- Most modern HPTLC quantitative analysis are performed in situ by measuring the zones of samples and standards using a chromatogram spectrophotometer usually called a densitometer or scanner with a fixed sample light beam in the form of a rectangular slit. Generally, quantitative evaluation is performed with the TLC Scanner III using winCATS software. It can scan the chromatogram in reflectance or in transmittance mode by absorbance or by fluorescent mode; scanning speed is selectable up to 100 mm/s. Spectra recording is fast. Calibration of single and multiple levels with linear or nonlinear regressions are possible.

10. Documentation

- Each developed plate is documented using digital documentation system under UV light at 254 nm, UV light at 366 nm, and white light. If a type of light does not produce usable information, that fact must be documented. If a plate is derivatized, images are taken prior and after derivatization.

1.7. ANALYTIC METHOD DEVELOPMENT AND VALIDATION[20-22]

Analytic method development and validation are continuous and interconnected activities conducted throughout the drug development process. Analytical methods are required to characterize drug substance and drug product composition during all phases of pharmaceutical

development. Early phase methods must support changes in synthetic routes and dosage form and elucidate the structures and levels of impurities. In later phases, goals change to the development of rapid and robust methods for release and stability evaluation that can be transferred to quality units. Analytic methods are intended to establish the identity, purity, physical characteristics and potency of the drugs that we use. Analytical method validation is the process of demonstrating that the analytical procedures are suitable for their intended use. According to FDA guideline, analytic method validation is a matter of establishing documented evidence that provides a high degree of assurance that the specified method will consistently provide accurate test results that evaluate a product against its defined specification and quality attributes. The validation process requires quality method development. Whereas validation can be a time-consuming process, methods should not enter the validation phase unless they are fully developed. The relationship of validation and method development can be

Observed as:

- When methods are properly developed, they can be readily validated.
- Validation does not make a method better or more efficient.
- A validated method does not necessarily imply that it meets all criteria of a properly developed method.
- Validation acceptance criteria should be based on method development experience.

Method Validation is required for the following reasons:

1. A new method is been developed.
2. Revision of established method.
3. When established methods are used in different laboratories and different analysts etc.
4. Comparison of methods.
5. When quality control indicates method changes.

Advantages of analytical method validation:

- The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.
- Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

Guidelines from the following sources provide a framework for performing validation.

- United states pharmacopoeia (USP)
- International conference on harmonization (ICH)
- Food and drug administration (FDA)
- Validation according to ICH Guidelines

Typical validation parameters are:

i) Accuracy
ii) Precision (Repeatability, Intermediate precision and Reproducibility)
iii) Linearity
iv) Range
v) Specificit
vi) Robustness
vii) System suitability testing
viii) Limit of detection (LOD) and Limit of quantitation (LOQ)

(*i*) Accuracy:

Definition: It expresses the closeness of agreement between the value which is accepted either asa conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined by recovery studies. The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range.

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value.

(*ii*) Precision :

Definition: It expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability: It expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. Repeatability must be tested from at least six replications measured at 100 percent of the test target concentration or from at least nine replications covering the complete specified range.

Intermediate precision: It expresses variations within laboratories, such as different days, different analysts, different equipment, and so forth. The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

Reproducibility: It expresses the precision between laboratories. The objective of reproducibility is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts.

(*iii*) Linearity:

Definition: Linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample.

It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/ separate weighing of synthetic mixtures of the drug product components, using the proposed procedure.

(i*v*) Range:

Definition: Range of an analytical procedure is the interval from the upper to the lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. For the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration should be tested/checked for range.

(*v*) Specificity:

Definition: It is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc.

(*vi*) Robustness:

Definition: It is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

(*vii*) System suitability testing:

Definition: The tests, based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. System suitability testing is an integral part of procedures.

(*viii*) Limit of detection and Limit of quantitation:

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value.

The quantitation limit of an individual analytical procedure is the lowest concentration of analyte in a sample, which can be quantitatively determined with a suitable level of precision and accuracy.

Several approaches for determining are possible, depending on whether the procedure is a non- instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation of the response and the slope.

The LOD and LOQ were estimated from the set of 5 calibration curves used to determine method linearity. Limit of detection and Limit of quantitation can be calculated by the following equation.

LOD = 3.3 (σ/S), LOQ = 10 (σ/S)

Where,

σ = Standard deviation of y-intercepts of regression lines

S =Slope of the calibration curve

Data Elements Required for Assay Validation

There are various analytical methods for the examination of pharmaceutical materials. Not all the characteristics referred above will need to be considered in all cases. Analytical method may be broadly classified as per WHO as follows.

Class A: tests design to establish identity, whether of bulk drug substances or of a particular ingredient in a finished dosage form.

Class B: Methods designed to detect and quantify impurities in a bulk drug substance or finished dosage form.

Class C: methods used to determine quantitatively the concentration of a bulk drug substance or of a major ingredient in a finished dosage form.

Class D: method used to assess the characteristics of finished dosage forms, such as dissolution profiles and content uniformity.

Table 1:Validation parameters that should be considered for different types of analytical procedures

Parameters	Class A	Class B		Class C	Class D
		Quantitative tests	Limit tests		
Accuracy		*		*	*
Precision		*		*	*
Robustness	*	*	*	*	*
Linearity & Range		*		*	*
Selectivity	*	*	*	*	*
LOD	*		*		
LOQ		*			

2.1 Literature Review

1. **A Validated High Performance Liquid Chromatographic (HPLC) Method for the estimation of Cefuroxime axetil P Santhosh Kumar *et al.*[23]**

A rapid and reproducible High Performance Liquid Chromatographic method has been developed for the estimation of Cefuroxime axetil in its pure form as well as in pharmaceutical dosage forms. Chromatography was carried out on an ODS C_{18} column (150 x 4.6 mm x 5 µm length), using a mixture of methanol and 0.01M potassium dihydrogen orthophosphate buffer (pH-2.0±0.05) (60:40 v/v) as the mobile phase at a flow rate of 0.8 mL/min and the detection was done at 248 nm. The method was developed and fully validated for the determination of Cefuroxime axetil. The retention time of the drug was 3.693 min. The method produced linear responses in the concentration range of 0.45 to 80 µg/mL of Cefuroxime axetil. Developed HPLC method was sensitive with LOD= 0.26 µg/mL and LOQ= 0.58 µg/mL. The method was successfully validated in accordance to ICH guidelines and was found to be reproducible for analysis of the drug in parenteral preparations.

2. **Development and validation of HPTLC Method for the estimation of Cefuroxime axeti - N J Shah *et al*[24]**

A simple, precise, accurate and rapid High Performance Thin Layer Chromatographic method has been developed and validated for the determination of Cefuroxime axetil in dosage form. The stationary phase used was precoated silica gel 60F254. The mobile phase used was a mixture of Chloroform: Methanol: Toluene (4:2:2 v/v/v). The detection of the spot was carried out at 290 nm. The method was validated in terms of linearity, accuracy, precision and specificity. The calibration curve was found to be linear between 300- 800 ng/spot. The LOD and LOQ of Cefuroxime was found to be 50 ng/spot and 100 ng/spot.

3. HPTLC Determination of Cefuroxime axetil and Ornidazole in Combined Tablet Dosage Form[25]

- **Poonam N Ranjane *et al.***

A new simple High-Performance Thin Layer Chromatographic method for determination of Cefuroxime axetil and Ornidazole in combined tablet dosage form is developed and validated. The separation is carried out on Merck precoated silica gel aluminium plate 60 F_{254} using toluene : *n*-butanol : triethylamine (8.5:2:0.5, v/v/v) as mobile phase. Quantitative determination of drugs is carried out by densitometric scanning of plates at 285 nm. The retention factor for Ornidazole and Cefuroxime axetil is found to be 0.51 ± 0.007 and 0.67 ± 0.009, respectively. The method is validated with respect to linearity, accuracy, precision, and robustness. Response is found to be linear in the concentration range of 100–500 ng/band for both Cefuroxime axetil and Ornidazole. The method has been successfully applied for the analysis of drugs in pharmaceutical formulation. The % assay is found to be 102.36 ± 0.775 and 101.00 ± 1.192 for Cefuroxime axetil and Ornidazole, respectively.

4. Development and validation of analytical method for the simultaneous estimation of Cefuroxime sodium and Potassium clavulanate in bulk and combined dosage form

- **J D Modi *et al.*[26]**

A Stability indicating RP-HPLC was developed and validated for determination of Cefuroxime sodium (CEF) and Potassium clavulanate (PCA). The RP-HPLC has shown adequate separation for Cefuroxime sodium and Potassium clavulanate from its degradation products. The separation was achieved on Hypersil BDS C_{18} column (250x4.6 mm, 5 µm particle size) using a mobile phase composition of Acetonitrile: Phosphate buffer pH4.5 (75:25) with a flow rate of 1ml/min. Injection volume 20µl and wavelength of detection was kept at 275 nm the retention time of Cefuroxime sodium and Potassium clavulanate were 3.06 and 7.63 min respectively. Linearity was observed over concentration range of 10-40 µg/ ml for Cefuroxime sodium and 6-20 µg/ml for Potassium clavulanate.

The mean recovery was found to be 100.08±0.68% and 99.95±0.67% for Cefuroxime sodium and Potassium clavulanate respectively The limit of detection was 0.34µg/ml and the limit of quantification was 0.112µg/ml for Cefuroxime sodium and the limit of detection was 0.097µg/ml and the limit of quantification was 0.292µg/ml for Potassium clavulanate.

5. Development and validation of UV simultaneous estimation of Cefuroxime axetil in bulk and Pharmaceutical dosage form.

- **Md Rezowanur Rahman et al [27]**

A rapid and sensitive UV-Visible spectroscopic method was developed for the estimation of Cefuroxime in pure and its pharmaceutical formulations. The method was based on the measurement of absorbance of Cefuroxime active moiety of Cefuroxime tablet at 277 nm using methanol as solvent. The absorbance was found to increase linearly with increase in concentration of Cefuroxime which was corroborated by correlation coefficient values. The standard solution of Cefuroxime obeyed Beer's law over the concentration range of 9.20– 27.60 µg/mL. The method is linear (from 9.20-27.60 µg/mL) with an R2 of 0.999, accurate (% recovery 100.56%) and precise (% RSD 0.316%). The method is specific and robust for Cefuroxime.

6. Simultaneous estimation of Cefuroxime axetil and Potassium clavulanate – analytical method development and validation

- **Pramod L I et al [28]**

A simple, rapid, sensitive spectrophotometric method has been developed for the simultaneous estimation of Cefuroxime axetil and Pot. clavulante in combined dosage form. The maximum absorbance of Cefuroxime axetil and Pot. clavulanate was measured in methanol at 284 nm and 271 nm. The calibration curve of both the drugs obeys the Beer's Law in the concentration range of 5-50 mcg/ml for Cefuroxime axetil and 1-30 mcg/ml for Pot. clavulanate with correlation coefficient value 0.999 and 0.998 at 284 nm and 271 nm respectively. The method was validated as per ICH guidelines. The results obtained in the method were in good agreement with the ICH parameters.

7. Spectrophotometric determination of Cefuroxime axetil from bulk and in its tablet dosage form

M V Shinde *et.al* [29]

Simple, rapid spectrophotometric method has been developed for estimation of Cefuroxime axetil from bulk drug and tablet dosage form by using 1-nitroso-2-napthol and Sodium hydroxide. The method is based on the formation of yellow-orange coloured complex with 1- nitroso-2-napthol having absorbance maxima at 424 nm. The Beer's Law is obeyed in the concentration range of 10-50 mcg/ml of the drug. The result of analysis of tablet formulation gave the percentage of label claim ±standard deviation as 99.17±1.57.

8. RP-HPLC method for the simultaneous determination of Cefuroxime axetil and Potassium clavlanate in tablet dosage form.

- **Mahima R S *et. al* [30]**

A simple, specific, accurate and precise RP-HPLC method for analysis of Cefuroxime axetil and Potassium clavulanate had been developed. Separation of drug was carried out on JASCO HPLC system with Hypersil Gold C_{18} column (250 mm×4.6 mm id) using 0.01 M Potassium dihydrogen phosphate: methanol (60:40 v/v) as mobile phase. Quantitation was carried out at a wavelength of 225 nm. Results were found to be linear in the concentration range of 5-50 mcg/ml for Cefuroxime axetil and 5-30 mcg/ml for Pot. clavulanate. Mean retension times for Pot. clavulanate and Cefuroxime axetil were found to be 2.573 and 8.293 respectively. Intra day variation as % RSD was 0.328 for Cefuroxime axetil and 0.382 for Potassium clavulanate. Inter day variation, as %RSD was 0.545 for Cefuroxime axetil and 0.552 foe Pot. clavulanate.
The percentage assay was found to be 100.976±0.439 for Cefuroxime axetil and 101.053±0.423for Pot. Clavulanate.

9. Simultaneous determination of Cefuroxime axetil and Potassium clavulanate in pharmaceutical dosage form by RP- HPLC

- **Pramod L I *et. al* [31]**

Chromatographic separation was achieved on reverse phase Microsorb-MV 100-5 C-18 (250x4.6mm, 5 µm) column with a mobile phase consisting of HPLC grade methanol:water in the ratio of 90:10 (v/v) at a flow rate of 1.0 mL/min

with UV detection at 230 nm. The retention time for Cefuroxime axetil and Potassium clavulanate were 2.46 and 3.33 minutes respectively. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness. The regression value for both the drugs was found to be 0.996 and 0.992, the SD and RSD values were found to be well within the acceptable limit of 2.0%

10. Development and validation of UV spectrophotometric method for the estimation of Linezolid in bulk and pharmaceutical formulation. [18]

- **P Prasanthi *et al*.[32]**

A simple, accurate, precise and sensitive UV spectrophotometric method was developed for the determination of Linezolid in bulk and pharmaceutical dosage form. The solvent used was 20% methanol and the wavelength corresponding to maximum absorbance of the drug was found at 251nm. Beers law was observed in the concentration range of 2-16µg/ml with correlation coefficient 0.999. The linear regression equation obtained by least square regression method was y=0.072X- 0.065, where y is the absorbance and x is the concentration of the pure drug solution. The method was validated for several parameters like accuracy, precision as per ICH guidelines.

11. Method development and validation of spectrophotometric method for the estimation of Linezolid in pure and tablet dosage form.

- **Sushama S *et al.*[33]**

A simple, specific and cost effective method for the estimation of Linezolid in tablets has been developed. Maximum wavelength was found to be 251 nm and validation was performed as per the ICH guidelines for linearity, accuracy, precision, LOD and LOQ. The method shows high sensitivity with linearity in the range of 1-6 µg/ml and shows a linear relationship between absorbance and concentration with a coefficient of correlation of 0.999. Precision of the method was good and the method was suitable for the analysis of pharmaceutical dosage form

12. RP-HPLC method development and validation for the analysis of pharmaceutical drugs – Linezolid.

- **V G Patel *et al.*[34]**

A simple, selective, linear, precise and accurate RP-HPLC method was developed and validated for rapid assay of Linezolid. Isocratic elution at a flow rate of 1.2 ml/min was employed on a symmetry C_{18} column at ambient temperature. The mobile phase consisted of acetonitrile: 0.1 M acetic acid 50:50 (v/v). The UV detection wavelength was at 254 nm. Linearity was observed in the concentration range if 100-140 ppm. Retension time for Linezolid was 3.3 min.

13. A validated stability-indicating LC method for the separation of enantiomer and potential impurities of Linezolid using polar organic mode

- **Satyanarayana Raju T *et al.*[35]**

This article explains a simple, precise, accurate stability-indicating LC method. It was developed for the determination of purity of Linezolid drug substance and drug products in bulk samples and pharmaceutical dosage forms in the presence of its impurities and degradation products. This method is capable of separating all the related substances of Linezolid along with the chiral impurity. This method can also be used for the estimation of assay of Linezolid in drug substance as well as in drug product. The method was developed using Chiral pak IA (250mm×4.6 mm, 5 mm) column. A mixture of acetonitrile, ethanol, n- butylamine and trifluoroaceticacid in 96:4:0.10:0.16 (v/v/v/v) ratio was used as a mobile phase. The eluted compounds were monitored at 254 nm. Linezolid was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. The degradation products were well resolved from main peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, limit of detection, limit of quantification, precision, linearity, accuracy, robustness and system suitability.

14. Spectrophotometric method development and validation for the estimation of Linezolid in tablet dosage Form

- **Gadhiya D T, Bagada H L [36]**

The difference absorption spectra of equimolar solution of Linezolid in 0.1M hydrochloric acid in sample cell relative to 0.1M sodium hydroxide in reference cell were taken. Absorption maxima was found 258.27 nm. The drug followed a linear relationship in the range of 4-20µg/ml; while the correlation coefficient was 0.999. The recovery was 100.01% ±0.17 the relative standard deviation for repeatability, intraday and interday was found to be less than 2%. These methods are found suitable for day to day analysis of linezolid in tablet dosage form.

15.Spectrophotometric method for the simultaneous estimation of Cefixime trihydrate and Linezolid in tablet dosage form

- **Patel S A *et al* [37]**

The method is based on the simultaneous equations for analysis of both the drugs using

0.05M potassium phosphate buffer pH 7.2 as solvent. Cefixime trihydrate has absorbance maxima at 287.20 nm and Linezolid has absorbance maxima at 250 nm. The linearity was obtained in the concentration range of 2- 22 µg/ml and 2-18 µg/ml for Cefixime and Linezolid, respectively.

16. Development and validation of stability indicating HPLC method for simultaneous estimation of Cefixime and Linezolid

- **Nidhi S P *et al* [38]**

For RP-HPLC , the separation was achieved by Phenomenex Luna C18(250×4.6 mm), 5 µm column using phosphate buffer(pH 7):methanol(60:40 v/v) as mobile phase with flow rate 1 ml/min. The wavelength selected for quantitation for Cefixime and Linezolid were 276 nm. The retention time of Cefixime and Linezolid were found to be 3.127 min and 11.986 min respectively. During forced degradation, the drug was exposed to hydrolysis (acid and base), H_2O_2, thermal and photo degradation. The percentage degradation was found to be 10-20% for both Cefixime and Linezolid in the given condition

17.Stability indicating RP-UPLC method development and validation for assay and content uniformity test of Linezolid with PDA detector.

- **Jebaliya H et al.** [39]

A method for the determination of content uniformity has been developed and validated for reducing analysis time and maintaining good efficiency. An isocratic separation of Linezolid was achieved on water Acquity BEH C18, 50×2.1 mm id, 1.7µm particle size column with a flow rate of 0.25 ml/min and using photodiode array detector to monitor the elute at 245 nm. A mobile phase consisting of methanol: water (50:50 v/v) to achieve good resolution and retention. The detector linearity was established by concentrations range of 1.5-80 µg/ml with a LOD and LOQ of 0.4 and 1.5 µg/ml respectively.

18. Development and validation of method for simultaneous estimation of Cefuroxime and Linezolid by

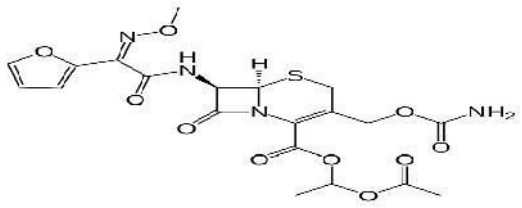
HPLC Kinjal a patel [40]

For RP-HPLC , the separation was achieved by Kromasil C-8 column using potassium dihydrogen ortho phosphate buffer(pH 4):methanol(60:40 v/v) as mobile phase with flow rate 0.9 ml/min. The wavelength selected for quantitation for Cefuroxime and Linezolid were 268 nm. The retention time of Cefuroxime and Linezolid were found to be 6.860 min and 8.840 min respectively. The detector linearity of Cefuroxime and Linezolid were found to be concentrations range of 2.5-12.5 µg/ml and 3-15 µg/ml respectively. The recoveries of Cefuroxime and Linezolid were found to be 101.6 - 101.90 % and 98.14 -101.15%respectively.

2.2. DRUG PROFILE

2.2.1. CEFUROXIME AXETIL

Molecular structure:

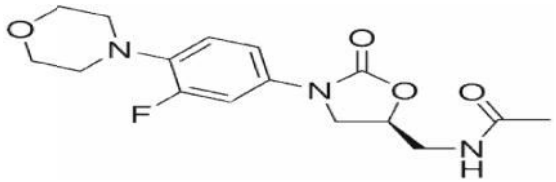


IUPAC Name: 1-acetyloxyethyl (6R,7R)-3-(carbamoyloxymethyl)-7-[[(2Z)-2-(furan-2-yl)-2-methoxyiminoacetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate

Molecular formula	:	$C_{20}H_{22}N_4O_{10}S$
Molecular weight	:	510.475 g/mol
CAS No.	:	64544-07-6
Melting point	:	173^0C.
Boiling point	:	804.2^0C
Density	:	1.76 g/cm^3
Flash point		440.2^0C.
Phase	:	Solid (STP).
Appearance	:	White to almost white crystalline powder.
Storage temperature	:	-20^0C freezer.
Solubility in water	:	Insoluble in water
Solvent solubility	:	Soluble in methanol, acetone,chloroform
Drug category	:	Anti Microbial
Brand names	:	oractil, Ceftum, altacef, cefuronat, ceftil, forcef

2.2.2. LINEZOLID[16]

Molecular structure:



IUPAC Name	:	N-((3-(3-fluoro-4-morpholinophenyl)-2-oxooxazolidin-5-yl)methyl)acetamide
Moleculer formula	:	$C_{16}H_{20}FN_3O_4$
Molecular weight	:	337.351 g/mol
CAS No	:	165800-03-3
Melting point	:	181.5-182.5^{0}C.
Phase	:	Solid (STP).
Appearance	:	Crystalline ,white to off white powder
Pka and pH	:	pka is 1.8 with Ph 4
Solubility in water	:	Soluble.
Solvent solubility	:	Soluble in Chloroform and alcohol, Insoluble in ether.
Stability	:	Stable, incompatible with strong oxidising agents
Shelf life	:	4 years.
Drug category	:	Anti Microbial
Brand names	:	linzomac,linospan,lizbid,megazolid,ozolid,linsomed,lizolid

Pharmaceutical dosage form used for the study consists of Cefuroxime axetil and Linezolid is available in the form of **ORACTIL-LZ** tablets. The dosage form contains 500mg of Cefuroxime axetil and 600mg of Linezolid

3.1 AIM AND OBJECTIVE

As per the literature review, there is no analytical methods reported for the estimation of Cefuroxime axetil and Linezolid in combined pharmaceutical dosage form by HPTLC. Various publications are available regarding the UV simultaneous estimation and RP-HPLC method development of Cefuroxime axetil and Linezolid, either alone or in combination with other drugs in pharmaceutical dosage form.

Hence,there is a need for suitable RP-HPLC and HPTLC Method for routine analysis of Cefuroxime axetil and Linezolid in the combined formulation.

The work was an attempts to develop simple, rapid, and sensitive analytical methods for the simultaneous estimation of Cefuroxime axetil and Linezolid in the combined formulation in accordance with ICH Q2B guidelines and to extend the method for routine analysis.

3.2 PLAN OF WORK

Present work is to develop and validate a new simple, rapid, and sensitive method for the simultaneous estimation of Cefuroxime axetil and Linezolid in the combined tablet dosage form by RP-HPLC and HPTLC method.

STEP-1: Study of physiochemical properties of the drug

STEP-2: Selection of chromatographic condition
Wave length,mobile phase,column,flow rate)

STEP-3: Optimization of the method

STEP-4: Study of the system suitability parameters

STEP-5: Validation of the proposed method

4. MATERIALS AND METHODS

4.1. MATERIALS AND METHODS FOR- HPLC

4.1.1. Chemicals and reagents

Table No.2: List of Chemicals and reagents

S.NO.	NAME	MANUFACTURER	GRADE
1.	Cefuroxime axetil working standard	Jivanta life sciences	-
2.	Linezolid working standard	Chemisol	-
3.	Oratil LZ	Macleods pharmaceuticals. Ltd	-
4.	Potassium dihydrogen phosphate	Merck	GR
5.	Sodium perchlorate	Merck	GR
6.	Perchloric acid	Merck	GR
7.	Ortho phosphoric acid	Merck	GR
8.	Methanol	Merck	HPLC
9.	Acetontrile	Merck	HPLC
10.	Water	Merck	HPLC
11.	0.45 µm Nylon filter	Axivia	S0761009
12.	0.45µm PVDF filter	Rankem	D004A07

4.1.2. Equipment/Instrument details

Table No.3: List of Equipment/Instrument details

S.NO.	INSTRUMENT NAME	MODEL
1.	HPLC system	Agilent 1220 Infinity LC(G4288C)
2.	Analytical balance	Shimadzu
3.	pH Meter	Thermo electron corporation orion 2 star
4.	Sonicator	Ultrasonic cleaner power sonic 420
5.	Vacuum oven	Wadegati
6.	Constant temperature water bath	Thermolab GMP

4.1.3. Analytical method development for the simultaneous estimation of Cefuroxime axetil and Linezolid by RP-HPLC.

A. Selection of wavelength

A solution of 100µg/mL of Cefuroxime axetil and Linezolid were prepared in methanol. The resulting solutions were scanned individually from 190 to 400 nm in UV-Visible spectrophotometer. Spectrums obtained are shown in Fig.No.9-11.

B. Selection of chromatographic condition

Proper selection of the method depends up on the nature of the sample (ionic/ ionisable / neutral molecule), its molecular weight and solubility. The drugs selected in the present study, were polar in nature. Thus reverse phase HPLC was selected for the initial separation because of its simplicity, suitability, ruggedness and its wider usage.

C. Initial separation condition

The mobile phase selected to elute the drug from the stationary phase was acetonitrile and phosphate buffer, because of its favorable UV transmittance, low viscosity and low back pressure.

D. Effect of buffer

Potassium di-hydrogen phosphate buffer was selected because better and higher intensity of response was obtained.

E. Effect of pH

The mobile phase pH was optimized using different pH, ranging from 2.0 to 3.0 (pH is adjusted with Ortho phosphoric acid), at a flow rate of 0.5 mL/min and symmetry Xterra C18 column as the stationary phase. The peak shape and resolution was observed at different pH.

F. Effect of ionic strength

The phosphate buffer was prepared in different strengths such as 0.01M, 0.025M, 0.05M of Potassium di-hydrogen phosphate at pH 2.8. The retention time was decreased by increasing the buffer strength. For the present study, the optimized mobile phase composition phosphate buffer of pH 2.8: acetonitrile (35:65v/v) was selected, because of the retention times of Cefuroxime axetil and Linezolid were effected due to slight change of ionic strength during analysis.

G. Effect of nature of stationary phase

The following stationary phases were used and the chromatograms were recorded.

1. Agilent zorbax SB C18 (4.6 x 150mm, 5μm)

2. Phenomex-kinetex-XDB C18 (4.6 x 100mm, 5μm)

3. Symmetry C8 (4.6 x 100mm, 5μm)

4. Symmetry XterraC18 column.

With Agilent zorbax SB C_{18} and Phenomex-kinetex-XDB-C18 the results obtained were not satisfactory because peak tailing was observed and also the resolution between the peaks was comparatively lesser than that with symmetry XterraC18. With XterraC18 column the peak shape and resolution observed were good. Therefore, Xterra C18 column was used for further studies.

Preparation of Placebo:

The amount of powdered inactive ingredient supposed to be present in 10 tablets were accurately weighed and transferred in to 100 ml volumetric flask, 70 ml of diluent was added and shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes and was diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for dilution. 0.6 ml of upper clear solution was transferred to a 100 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 μm filter before injecting into HPLC system.

Preparation of Phosphate buffer:

7.0 grams of KH_2PO_4 was weighed into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water. The flask was shaken until the particles get dissolved and volume was made up to the mark with Water. The pH was adjusted to 2.8 with ortho phosphoric acid.

TRIALS

Trial 1:

Method development for the drugs was initiated based on the individual chemical charecteristics and their methods given in individual journals.

Mobile phase: Methanol : Acetonitrile (50:50v/v)

Diluent: methanol

Chromatographic conditions

Flow rate	**:**	1.5 ml per min
Column	**:**	Agilent zorbax SB C18(4.6 x 150mm, 5μm)
Detector wavelength	**:**	284 nm
Column oven	**:**	Ambient
Injection volume	**:**	20 μl

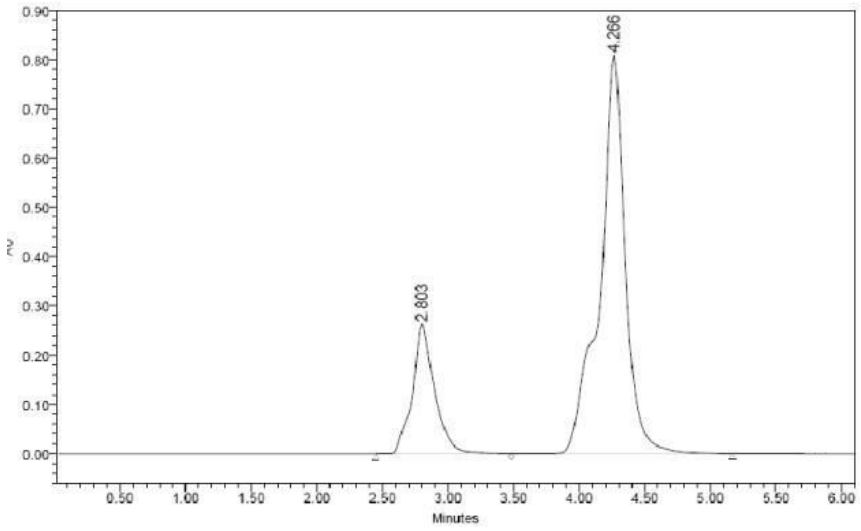


Fig.No.3: Chromatogram for Trail 1

Observation: Theoretical plates were less and the system suitability was failed. The Chromatogram for trial 1 is shown in Fig.No.6.

Result: Separation occurred below 6 min. System suitability was failed. Fronting of the peaks was seen.

Trail 2:

Inorder to improve resolution and remove fronting of the peak and avoid unwanted peaks interfering, column and mobile phase was changed and again the same experiment was performed.

Mobile phase: Phosphate buffer (pH 3.5): Methanol (50:50v/v)

Diluent: Methanol

Chromatographic conditions

Flow rate : 1.5 ml per min

Column : Phenomex-kinetex-XDB-C_{18} (4.6 x 100mm, 5μm)

Detector wavelength : 284 nm

Column oven : Ambient

Injection volume : 20 μl

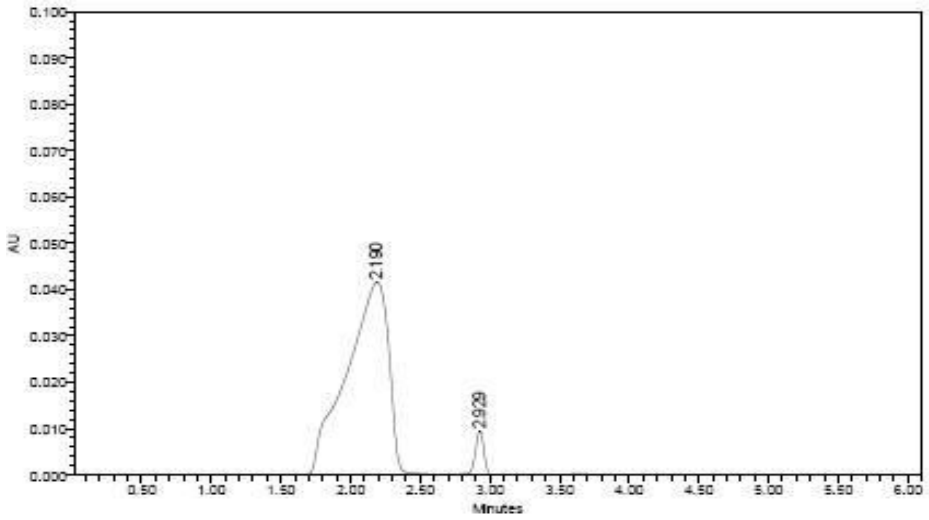


Fig.No.4: Chromatogram for trial 2

Observation: Resolution was less and the system suitability was failed due to the poor column performance. The Chromatogram for trial 2 is shown in Fig.No.7.

Result: Blunt peaks with less resolution were obtained due to the poor column performance. Hence experiment was again repeated by changing the column.

TRIAL-3

Inorder to avoid poor resolution column was changed and flow rate was decreased.

Mobile phase: Phosphate buffer (pH 2.8): Methanol (50:50v/v)

Diluent: Methanol.

Chromatographic conditions

Flow rate : 1.00 ml per min

Column : Symmetry C8(4.6 x 100mm, 5μm)

Detector wavelength : 284 nm

Column oven : Ambient

Injection volume : 20 μl.

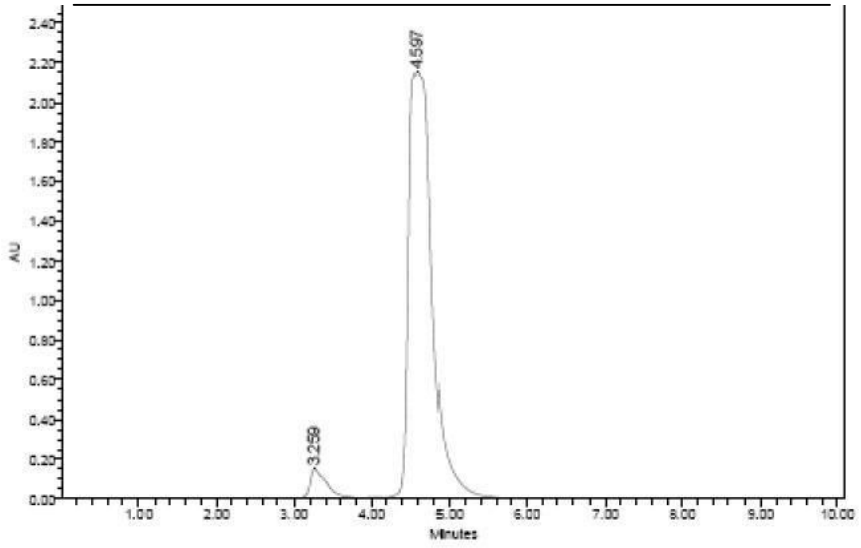


Fig.No.5: Chromatogram for trial 3

Observation: peaks were eluted but with less resolution, hence column was again changed. The chromatogram for trial 3 is shown in Fig.No.8.

Result: peaks were eluted but with less resolution, hence column was again changed.

OPTIMIZED METHOD

Optimized method for the simultaneous estimation of Cefuroxime axetil and Linezolid by RP-HPLC was finally achieved by using the following chromatographic conditions.

Chromatographic conditions

Flow rate : 1 ml per min

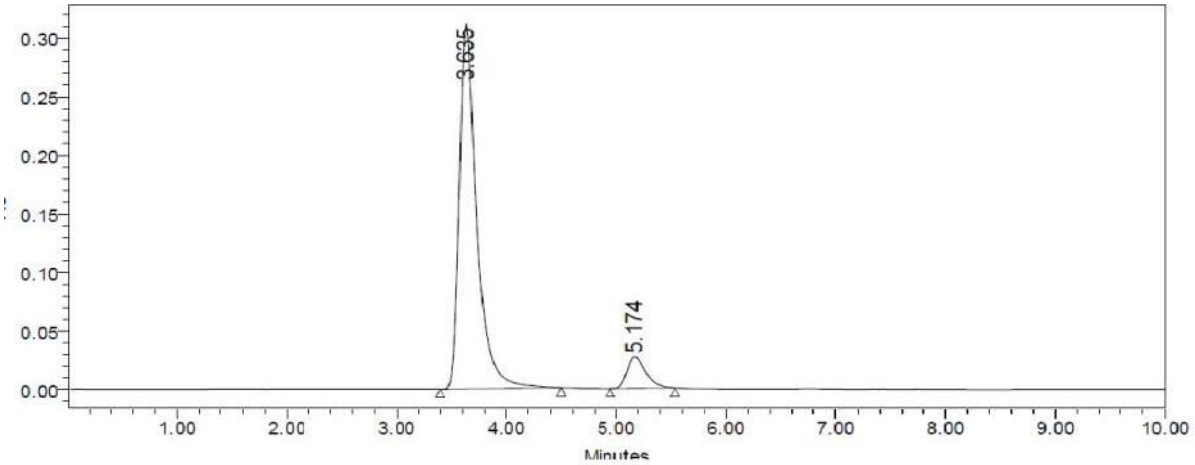
Column : C18 Symmetry (4.6 x 150mm, 5μm, Make:Xterra) .

Detector wavelength : 284 nm

Column oven : Ambient

Injection volume : 20 μl

Run time : 10 min



Procedure

Preparation of mobile phase: Mixture of above buffer 350 ml (35%) and 650 ml of acetonitrile HPLC (65%) were mixed and degassed in ultrasonic water bath for 5 minutes and filtered through 0.45 μ filter under vacuum filtration.

Diluent Preparation: Mobile phase was used as Diluent.

Preparation of standard solution (Mixed standard) : 10 mg of Cefuroxime axetil and 10mg of Linezoild working standards were accurately weighed and transferred into a 100ml clean dry volumetric flask add about 70ml of diluent was added and sonicated to dissolve it completely and the volume was made up to the mark with the same solvent. (Stock solution) Further 1.2ml of Cefuroxime axetil & 3ml of Linezolid was pippeted from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of sample solution

10 Tablets of Cefuroxime axetil and Linezolid were weighed and powdered in glass mortar. The powder equivalent to the amount of active ingredient present in 10 tablets (156.8mg) was transferred into a 100 ml clean dry volumetric flask, 70 ml of diluent was added to it and was shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution). 0.6ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 μm filter before injecting into HPLC system.

Test Procedure

20 µl of the standard, sample, blank and placebo preparations in duplicate were injected separately into HPLC system and the peak responses for Cefuroxime axetil and Linezolid were measured. The quantities in mg of Cefuroxime axetil and Linezolid were calculated per tablet taken.The developed RP-HPLC method for the simultaneous estimation of Cefuroxime axetil and Linezolid was carried out on

XterraC_{18}, 150 mm × 4.6 mm, 5 μm column in isocratic mode using mobile phase composition of phosphate buffer (pH 2.8 with ortho phosphoric acid) : acetonitrile [35 : 65, v / v] with flow rate of 1 ml / min at 284nm. The asymmetric factor was found to be 1.58 for Cefuroxime axetil and 1.47 for Linezolid

Calculation: The amount of drug present was calculated by using the following formula:

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where

AT = average area counts of sample preparation.

As = average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = Label claim of drug in mg/ml.

The individual chromatograms of Cefuroxime axetil and Linezolid, standard, sample, blank chromatograms for optimized method are shown in Fig.No.12-16.Results are tabulated in Table .No.5.

4.3. METHOD VALIDATION

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. According to ICH Q2B guidelines, typical analytical performance characteristics that should be considered in the validation of the types of methods are:

1. Specificity.
2. Linearity.
3. Accuracy.
4. Precision.
5. Limit of detection.
6. Limit of quantification.
7. Robustness.
8. System suitability.

1. SPECIFICITY

A) Cefuroxime axetil and Linezolid identification

Solutions of standard and sample were prepared as per test procedure and injected into the HPLC system. The recorded chromatograms are shown in Fig.No.17 and 18.

Acceptance criteria

Chromatogram of standard and sample should be identical with near retention time.

B) Placebo interference

A study to establish the interference of placebo was conducted. A sample of placebo was injected into the HPLC system as per the test procedure. The chromatogram of placebo is shown in Fig.No.19.

Acceptance criteria

- Chromatogram of placebo should not show any peak at the retention time of analyte peak.
- There is no interference due to placebo at the retention time of analyte. Hence the method is specific.

C) Blank interference

A study to establish the interference of blank was conducted. Diluent was injected into HPLC system as per the test procedure. The chromatogram of blank is shown in Fig.No.20.

Acceptance criteria

Chromatogram of blank should not show any peak at the retention time of analyte peak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

2. LINEARITY

Preparation of stock solution

10 mg of cefuroxime axetil and 10mg of linezolid working standards were accurately weighed and transferred into a 100ml clean dry volumetric flask and about 70ml of diluent was added and sonicated to dissolve it completely and the volume was made up to the mark with the same solvent.

Preparation of Level – I (4ppm of Cefuroxime axetil and 10ppm of Linezolid)

0.4ml and 1 ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – II (8ppm of Cefuroxime axetil and 20ppm of Linezolid)

0.8ml and 2 ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – III (12ppm of Cefuroxime axetil and 30ppm of Linezolid)

1.2ml and 3 ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – IV (16ppm of Cefuroxime axetill and 40ppm of Linezolid)

1.6ml and 4 ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – V (20ppm of Cefuroxime axetill and 50ppm of Linezolid

2.0ml and 5 ml of stock solution were taken in 10ml of volumetric flask diluted up to the mark with diluent.

Procedure

Each level solution was injected into the chromatographic system and the peak area was measured. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated.

The linearity of the method was demonstrated over the concentration range of 10-50 µg / ml. Aliquots of10, 20, 30, 40 and 50 µg / ml were prepared from sample solution and labeled as solution 1, 2, 3, 4 and 5 respectively. The solutions were injected in to HPLC system as per test procedure. The Chromatograms are shown in Fig.No.21-25.Acalibration curve was plotted for concentration v/s peak area and is shown in the Fig.No.26 and 27.The results are tabulated in Table No. 6 and 7.

Acceptance criteria

- Correlation Coefficient should be not less than 0.9990.
- % RSD of peak area's for Solution 1, 2, 3, 4 and 5 should be not more than 2.0 %.

3. ACCURACY

Assay was performed in triplicate for various concentrations of cefuroxime axetil and linezolid equivalent to 50, 100, and 150 % of the standard amount was injected into the HPLC system per the test procedure.

Preparation of Standard stock solution:

10 mg of cefuroxime axetil and 10mg of linezolid working standards were accurately weighed and transferred into a 100ml clean dry volumetric flask about 70ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent (Stock solution).Further 1.2ml of cefuroxime axetil and 3 ml of linezolid of the above stock solutions were pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation Sample solutions:

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

5.0 mg of cefuroxime axetil and 5.0mg of linezolid working standards were accurately weighed and transferred into a 10ml clean dry volumetric flask about 7ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent (Stock Solution).Further 1.2ml of cefuroxime axetil and 3 ml of linezolid of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

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

10 mg of cefuroxime axetil and 10 mg of linezolid working standards were accurately weighed and transferred into a 10ml clean dry volumetric flask about 7ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent (Stock solution).Further 1.2ml of cefuroxime axetil and 3ml of linezolid of the above stock solutions were pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

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

15.3mg of cefuroxime axetil and 14.8 mg of of linezolid working standards were accurately weighed and transferred into a 10ml clean dry volumetric flask , about 7ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent (Stock solution).Further 1.2ml of cefuroxime axetil l and 3ml of of linezolid of the above stock solution was pipetted in to a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure

Standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected in to HPLC system. Amount found and amount added for cefuroxime axetil and of linezolid, individual recovery and mean recovery values were also calculated. The average % recovery of cefuroxime axetil and linezolid,was calculated and the Chromatograms are shown inFig.No.28-31.Results are tabulated inTable No.8 and 9.

Acceptance criteria

The mean % recovery of the cefuroxime axetil and linezolid, at each spike level should be not less than 98.0 % and not more than 102.0 %.

4. PRECISION

a) REPEATABILITY

Preparation of stock solution (solution A)

10 mg of cefuroxime axetil and 10mg of linezolid, working standards were accurately weighed and transferred into a 100ml clean dry volumetric flask about 70ml of diluent was added and sonicated to dissolve it completely and volume was made up to the

mark with the same solvent. Further 1.2ml of cefuroxime axetil and 3ml of linezolid, of the solution A was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure

The standard solution was injected for five times and the area was measured for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.The chromatograms are shown in Fig.No.32.The results are tabulated in Table No.10 and 11.

Acceptance criteria

- All individual assays of cefuroxime axetil and linezolid, tablets should be within 98 % - 102 %.
- Relative standard deviation of % Assay results should not be more than 2.0.

b) INTERMEDIATE PRECISION (analyst to analyst variability): To evaluate the intermediate precision (also known as the ruggedness) of the method precision was performed on different days by using different columns of same dimensions.

Preparation of stock solution (solution A)

10 mg of cefuroxime axetil and 10mg of linezolid, working standards were accurately weighed and transferred into a 100ml clean dry volumetric flask about 70ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent. Further 1.2ml of cefuroxime axetil and 3ml of linezolid of the solution A was pipetted out in to a into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure

The standard solution was injected for five times and the areas for all five injections were measured in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.Two analysts as per test method conducted the study. Chromatograms are shown in Fig.No.33. For analyst-1 refer precision (repeatability) results and the results for analyst-2 are tabulated inTable No.12-15.

Acceptance criteria

- All the individual assays of cefuroxime axetil and linezolid tablets should be within 98 % - 102 %.
- Relative standard deviation of % assay results should not more than 2.0 % by both the analysts.

5. LIMIT OF DETECTION (LOD)

Cefuroxime axetil

Preparation of 12µg/ml solution:10mg of cefuroxime axetil working standard was accurately weighed and transferred to100ml clean dry volumetric flask, about 70ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent (Stock solution).Further 1.2ml of the above stock solution was pipetted into a 10mlvolumetric flask and diluted up to the mark with diluent.

Preparation of 0.25% solution at specification level (0.003µg/ml solution)

Further 1ml of the above stock solution was pipetted into a 10ml volumetric flask anddiluted up to the mark with diluent. Further 1ml of the above stock solution waspipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

0.25mlof 1µg/ml solution was pipetted into a 10 ml of volumetric flask and diluted up to themark with diluent.

Linezolid

Preparation of 30µg/ml solution

10mg of linezolid working standard was accurately weighed and transferred into a100ml clean dry volumetric flask, about 70ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent (Stock solution). Further 3ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 0.3% solution at specification level (0.09 µg/ml solution)

Further1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluents. 0.3ml of 1µg/ml solution was pipetted into a 10 ml of volumetric flask and diluted up to the mark with diluent. Chromatograms which were recorded are shown in Fig.No.34 and 35.

The LOD is determined by the formula

LOD =S/N

Where

N = Average Baseline Noise obtained from Blank

S = Signal Obtained from LOD solution (0.25% of target assay concentration)

Acceptance Criteria: S/N Ratio value shall be not more than 3 for LOD solution.

6. LIMIT OF QUANTIFICATION (LOQ)

Cefuroxime axetil

Preparation of 12µg/ml solution

10mg of cefuroxime axetil working standard was accurately weighed and transferred into a 100ml clean dry volumetric flask, about 70ml of diluent was added and sonicated to dissolve it completely and the volume was made up to the mark with the same solvent (Stock solution). Further 1.2ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of solution at specification level (0.012µg/ml solution)

Further 1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.1ml of solution was pipetted into a 10 ml of volumetric flask and diluted up to the mark with diluent.

Linezolid

Preparation of 30µg/ml solution

10mg of Linezolid working standard was accurately weighed and transferred into100ml clean dry volumetric flask, about 70ml of diluents was added and sonicated to dissolve it completely and the volume was made up to the mark with the samesolvent (Stock solution).Further 3ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 1.0% solution at specification level (0.3µg/ml solution):

Further 1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent. 1.0ml of 1µg/ml solution was pipetted into a 10 ml of volumetric flask and diluted up to the mark with diluent. Chromatograms which were recorded are shown in Fig.No.36 and 37.

LOQ is determined by the following formula:

LOQ =S/N

where

N = Average Baseline Noise obtained from Blank.

S = Signal Obtained from LOQ solution (1% of target assay concentration).

Acceptance Criteria: S/N Ratio value shall be 10 for LOQ solution.

7. ROBUSTNESS

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like flow rate and mobile phase composition, temperature variations which may differ but the responses were still within the specified limits of the assay.

a) Effect of variation of flow rate

A study was conducted to determine the effect of variation in flow rate. The flow rate was varied at 0.4ml/min to 0.6 ml/min. Standard solution 12ppm of cefuroxime axetil and 30ppm of linezolid was prepared and analysed using the varied flow rates along with method flow rate. The results are summarized. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.The method is robust only in less flow condition. The effect of variation of flow rate was evaluated. The Chromatograms are shown in Fig.No.38 and 39. The results are tabulated in the Table No.16 and 17.

Acceptance criteria

- The tailing factor for Cefuroxime axetil and Linezolid should not be more than 2.0 for Variation in flow.
- The % RSD of asymmetry and retention time for Cefuroxime axetil and Linezolid should not be more than 2.0 % for variation in flow.

b) Effect of variation of mobile phase composition

A study was conducted to determine the effect of variation in mobile phase ratio by changing the ratio of mobile phase. The organic composition in the Mobile phase was varied from 55% to 70%.

Standard solution 12 µg/ml of cefuroxime axetil and 30µg/ml of linezolid was prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method. Standard solution was prepared and injected into the HPLC system.The chromatograms which are recorded are shown in Fig.No.40and 41. The retention time values are measured and are tabulated in Table No.18 and 19.

Acceptance criteria

- Tailing Factor of cefuroxime axetil and linezolid drugs should not be more than 2.0 for Variation in composition of mobile phase.
- The % RSD of tailing factor and retention times of cefuroxime axetil and linezolid drugs should be not more than 2.0 for Variation in composition of mobile phase.

8. SYSTEM SUITABILITY

Sample solution of cefuroxime axetil and linezolid were injected three times into HPLC system as per test procedure. The system suitability parameters were evaluated from standard chromatograms obtained, by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from three replicate injections.

Acceptance criteria

- The % RSD for the retention times of principal peak from 3 replicate injections of each Standard solution should be not more than 2.0 %
- The number of theoretical plates (N) for the cefuroxime axetil and linezolid peaks should be not less than 2000.
- The Tailing factor (T) for the cefuroxime axetil and linezolid peaks should be not more than 2.0.

From the system suitability studies it was observed that all the parameters were within limit. Hence it was concluded that the instrument, reagents and column were suitable to perform the assay. Chromatogram is shown inFig.No.42. The results are tabulated in Table No.20 and 21.

4.2. MATERIALS AND METHODS FOR HPTLC

4.2.1 MATERIALS

The reagents and chemicals used for the experimental works are as follows:

- Reference Standards of Cefuroxime axetil and Linezolid.
- Marketed combination product of Cefuroxime axetil and Linezolid.
- (Oratil LZ containing Cefuroxime axetil 500 mg and Linezolid 600 mg marketed by Macleods Pharma Private Limited .)
- Methanol HPLC grade obtained from Merck Specialities (P) Ltd, Mumbai.
- Toluene HPLC grade obtained from Merck Specialities (P) Ltd, Mumbai.
- Hexane HPLC grade obtained from Merck Specialities (P) Ltd, Mumbai.

4.3.2 EQUIPMENT USED

Application mode : CAMAG Linomat IV Development mode

CAMAG Twin Trough chamber Scanner : TLC scanner with WINCATS software

Visualization : CAMAG UV Cabinet

Quantification : CAMAG Video Densitometer

Stationary phase : TLC plates (20 × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany

4.3.3.METHODOLOGY ADOPTED

1. Preparation of standard solutions of Cefuroxime axetil (CEF) and Linezolid (LIN)
2. Development of solvent system.
3. Development of chromatogram.
4. Determination of Rf values of CEF and LIN.
5. Preparation of calibration curves of CEF and LIN and estimation of CEF and LIN in dosage form.
6. Validation of the proposed method.

1. Preparation of standard solutions

a) Stock solution of Cefuroxime axetil RS in methanol

Weighed accurately 50 mg Cefuroxime axetil RS and transferred to a 50 ml standard flask. It was dissolved in HPLC grade methanol and made up to the volume. This had a concentration of 1 mg/ml.

b) Stock solution of Linezolid RS in methanol

Weighed accurately 50 mg of Linezolid RS and transferred to a 50 ml standard flask. It was dissolved in HPLC grade methanol and made up to the volume. This solution had a concentration of 1 mg/ml.

c) Preparation of standard drug mixture

50 mg of Cefuroxime axetil RS and 60 mg of Linezolid RS were weighed separately and transferred into a 100 ml standard flask. The drug mixture was allowed to dissolve in sufficient quantity of methanol by shaking for 15 min and the volume was made up to the mark with methanol to obtain a mixture with concentration of 500 µg/ml of Cefuroxime axetil and 600 µg/ml of Linezolid.

2. Development of solvent system

The mobile phase was selected based on the polarity of analytes (Cefuroxime axetil and Linezolid) and adsorption properties of silica gel plates. The solubility of drug played a significant role in the selection of suitable solvent system.

The suitable solvent system was selected by a series of trial and error process. Different solvent systems were used in different proportions and the summary is listed in Table 21.

Table 22: Solvent system selection trial and error data.

Sl. No.	Solvent system	Ratio	Inference
1	Toluene	---	A very Little movement
2	Methanol	---	Moved up to solvent front
3	Toluene : Methanol	5 : 5	Moved with no resolution
4	Toluene : Ethyl acetate	5 : 5	No movement
5	Toluene : Chloroform	5 : 5	No movement
6	Toluene : Methanol	1 : 9	Moved with a little resolution
7	Toluene : Methanol	2 : 8	Moved with a little resolution
8	Toluene: Chloroform : Methanol	2 : 2 : 6	No movement for spots
9	Toluene : Ethyl acetate : Methanol	2 : 2 : 6	No movement for spots
10	Hexane: Toluene: Methanol	2 : 2 : 6	Better resolution

4.3.4. Optimization of mobile phase

Methanol: Toluene: Hexane mobile phase system was optimized by changing the ratio of solvents. Table 22 shows different ratios of solvents tried.

Table 23: Optimization of mobile phase data

1	Methanol : Toluene : Hexane	1 : 8 :1
2	Methanol : Toluene : Hexane	2 : 6 : 2
3	Methanol : Toluene : Hexane	2 : 5 : 3
4	Methanol : Toluene : Hexane	2 : 6 : 2
5	Methanol : Toluene : Hexane	3 : 3 :4

Methanol : Toluene : Hexane (2 : 5 : 3 , v/v/v) was chosen as the mobile phase, which gave a chromatogram with good resolution for Cefuroxime axetil and Linezolid.

3. Development of chromatogram.

- **Selection of chromatographic layer**

  HPTLC pre-coated plates of silica gel G 60 F_{254} were employed for the spotting of standard solutions.

- **Preparation of mobile phase and saturation of Twin trough chamber**

  Mobile phase (Methanol : Toluene : Hexane in the ratio, 2 : 5 : 3 v/v/v) was freshly prepared and transferred into a clean and dried twin trough chamber. The chamber was then allowed to saturate for 30 minute.

- **Activation of plate and sample application**

  Three tracks were selected on the activated pre coated HPTLC plate and spotting was done by using CAMAG Linomat IV sample applicator in the form of bands. Cefuroxime axetil standard was applied on the first track, and Linezolid standard was on the second track. Volume of sample application was selected according to the volatility of solvent used for preparing the sample solution. The applied band was sharp when the volume was 2 µl. A band width of 4 mm was selected for the entire experiment.

  The following manual adjustments were done in the Linomat applicator

Plate size	: 10 x 6 cm
Start position	: 12 mm
Band width	: 4 mm
Application volume	: 2 µl
Flow rate	: 2 µl/sec
Space	: 12 mm

  After application the plates was taken out and the position of spots were visualized and confirmed under UV cabinet at 254 nm.

➢ **Development of spot**

The plate was developed in the saturated twin trough chamber containing the mobile phase. The plate was dried after development and viewed under UV cabinet to evaluate the spots obtained. The spots were uniform and there was no tailing.

4. Determination of Rf values of Cefuroxime axetil and Linezolid

➢ **Detection and visualization**

The developed plate was mounted on the CAMAG HPTLC scanner IV and scanned from

200-400 nm. The spots showed good response at 254 nm. The Rf values are furnished in

Table 23 and the chromatograms were displayed in Figure 27 and Figure 28.

Table 24: Rf values of drug under study

Drug	Rf
Cefuroxime axetil	0.21
Linezolid	0.30

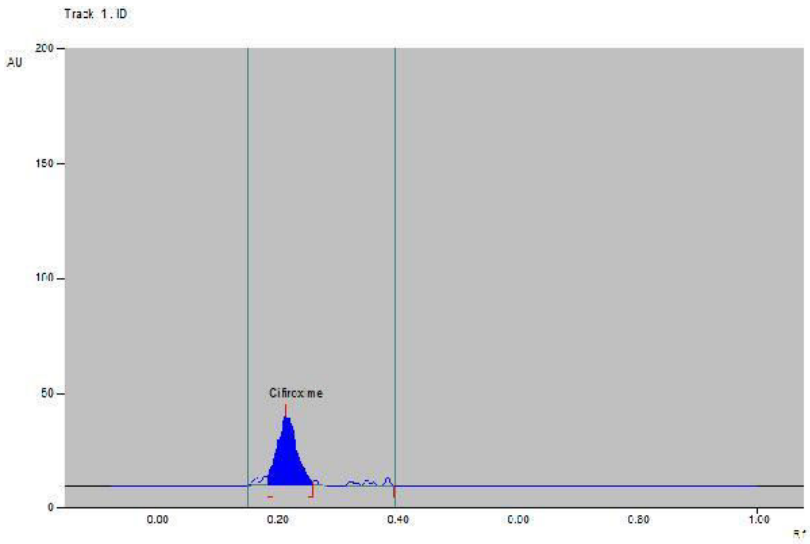


Figure 45: Chromatogram of Cefuroxime axetil with Rf 0.21

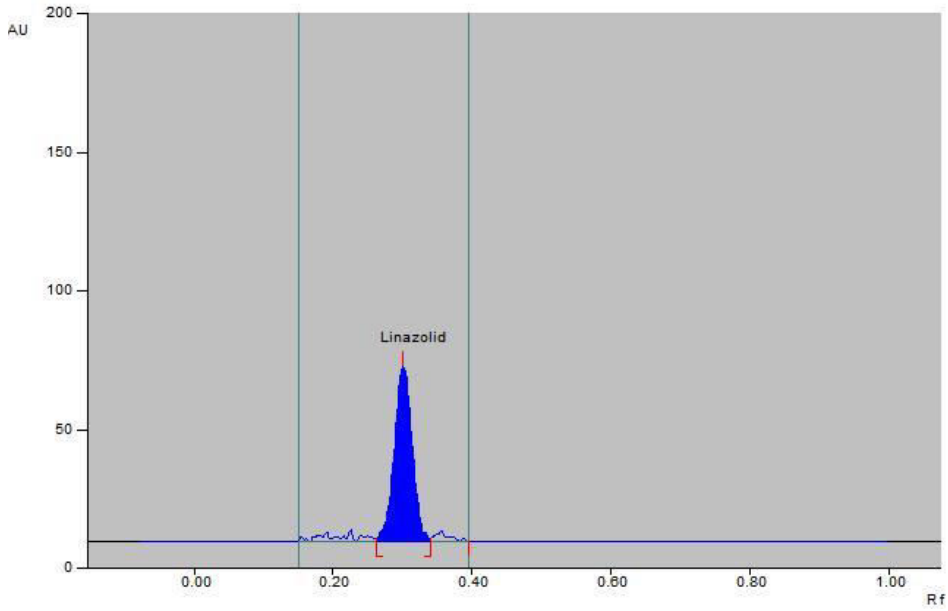


Figure 46: Chromatogram of Linezolid with Rf 0.30

5. Preparation of calibration curves of Cefuroxime axetil and Linezolid and analysis of combined tablet dosage form.

A. Preparation of standard solutions

- **Standard solutions of Cefuroxime axetil RS in methanol**

Weighed accurately 50 mg of Cefuroxime axetil RS and transferred to a 50 ml standard flask. It was dissolved in HPLC grade methanol and made up to the volume. This solution had a concentration of 1000 µg/ml.

From the above solution 0.5 ml, 1 ml, 1.5 ml, and 2 ml were pipetted out into four numbered

10ml standard flask and volume was made up to the mark with methanol to get a concentration of 50 µg/ml, 100 µg/ml, 150 µg/ml, and 200 µg/ml.

- **Standard solution of Linezolid in methanol**

Weighed accurately 50 mg of Linezolid RS and transferred to a 50 ml standard flask. It was dissolved in HPLC grade methanol and made up to the volume. This solution had a concentrantion of 1000 µg/ml.

From the above solution 0.5 ml, 1 ml, 1.5 ml and 2 ml were pipetted out into four numbered

10 ml standard flask and the volume was made up to the mark with methanol to get a concentration of 50 µg/ml,100 µg/ml,150 µg/ml and 200 µg/ml.

- **Preparation of standard drug mixture**

50 mg of Cefuroxime axetil RS and 60 mg of Linezolid RS were weighed separately and transferred into a 100ml standard flask. The drug mixture was allowed to dissolve in sufficient quantity of methanol by shaking for 15 min and the volume was made up to the mark with methanol. From the resultant solution, accurately pipetted out 1.0 ml into a 10 ml standard flask and made up to the mark with methanol to obtain a mixture with concentration of 50 µg/ml of Cefuroxime axetil and 60 µg/ml of Linezolid.

B. Preparation of sample solution

Details of Analysed Dosage Form

Trade name	:	Oratil LZ
Label claim	:	Cefuroxime axetil 500 mg and Linezolid 600 mg
Mfd By	:	Macleods Pharma

Twenty tablets of Oratil LZ were weighed; average weight of one tablet was determined and finely powdered with the help of mortar and pestle. A quantity of powdered tablet equivalent to 50 mg of Cefuroxime axetil (which contains about 60 mg of Linezolid) was accurately weighed, transferred to a stoppered flask and extracted with 20 ml of methanol initially by shaking vigorously for 15 minutes. The solution was transferred to a 100 ml standard flask through a Whatman No. 1 filter paper. The residue was further

extracted twice with 10ml of methanol and transferred to the same standard flask through the same filter paper. The volume was finally made up to 100 ml with methanol. From the above solution accurately pipetted out 1.0 ml and transferred to 10 ml standard flask and then made up to the mark with methanol. The resulting solution had a concentration of 50 µg/ml of Cefuroxime axetil and 60 µg/ml of Linezolid as per label claim.

C. Development of chromatogram

- **Selection of chromatographic layer**

  HPTLC pre-coated plates of silica gel G 60 F_{254} were employed for the spotting of standard solutions.

- **Preparation of mobile phase and saturation of twin trough chamber**

  Mobile phase containing Methanol : Toluene : Hexane in the ratio 2 : 5 : 3 , v/v/v was freshly prepared and transferred into a clean and dry twin trough chamber. The chamber was then allowed to saturate for 30 minute.

- **Activation of plate and sample application**

  Seventeen tracks were selected on the activated pre coated HPTLC plate and spotting was done by using CAMAG Linomat IV automatic sample applicator in the form of bands. Cefuroxime axetil standard were applied on the first four tracks, and Linezolid were applied on the next four tracks. Standard drug mixture was applied on track number nine and sample was applied on track number ten.

  The following manual adjustments were done in the Linomat applicator

  Plate size : 20x10 cm
  Start position : 12 mm
  Band width : 4 mm
  Application volume : 2 µl
  Flow rate : 2 µl/sec
  Space : 9 mm

  After application the plate was taken out and the position of spots were visualized and confirmed under UV cabinet at 254.

- **Development of spot**

  The plate was developed in the saturated twin trough chamber containing the mobile phase. The plate was dried after development and viewed under UV chamber to evaluate the spots obtained. The spots were uniform without tailing.

- **Scanning and integration of chromatogram**

  The developed plate was mounted on the CAMAG HPTLC scanner IV and scanned at 254 nm. The results are furnished in Table 24. The calibration graphs of concentration v/s peak height and concentration v/s peak area were plotted and shown in fig 29 to 32. The overlay spectrum is shown in figure 33. The developed plate is shown in figure 34. The chromatograms of standards are shown in figure 35(a-h). Chromatograms of standard and sample are displayed in figure 36 and 37.

Table 25: Chromatogram analysis data

Track	Drug	Concentration ng/band	Rf	Peak height	Peak area (A U)
1	CEF	100	0.21	29.91	728.77
2	CEF	200	0.21	58.3	1340.68
3	CEF	300	0.21	85.17	2014.54
4	CEF	400	0.21	112.41	2655.28
5	LIN	100	0.30	64.04	1233.72
6	LIN	200	0.30	114.04	2328.45
7	LIN	300	0.30	158.23	3190.3
8	LIN	400	0.30	197.75	4162.51
16	Standard drug mixture		0.21	29.82	724.86
16			0.30	75.67	1474.89
17	Sample		0.21	30.11	722.56
17			0.30	75.28	1475.20

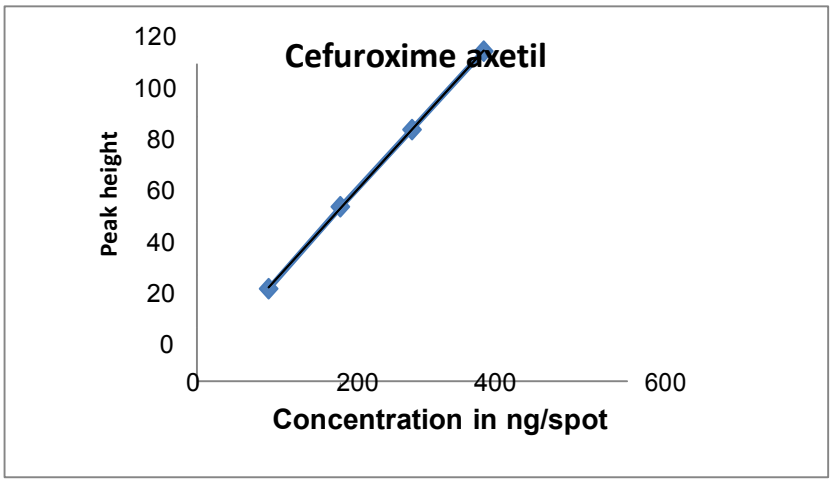


Figure 47: Calibration graph of Cefuroxime axetil [Concentration v/s Peak height]

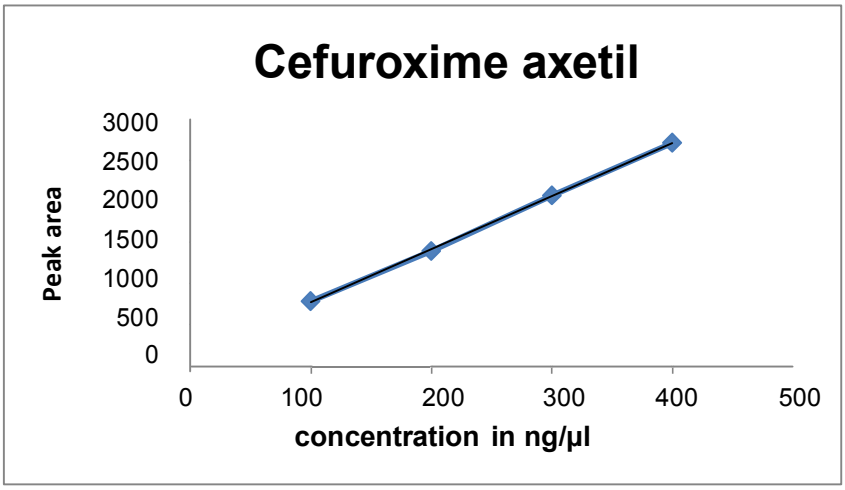


Figure 48: Calibration graph of Cefuroxime axetil [Concentration v/s Peak area]

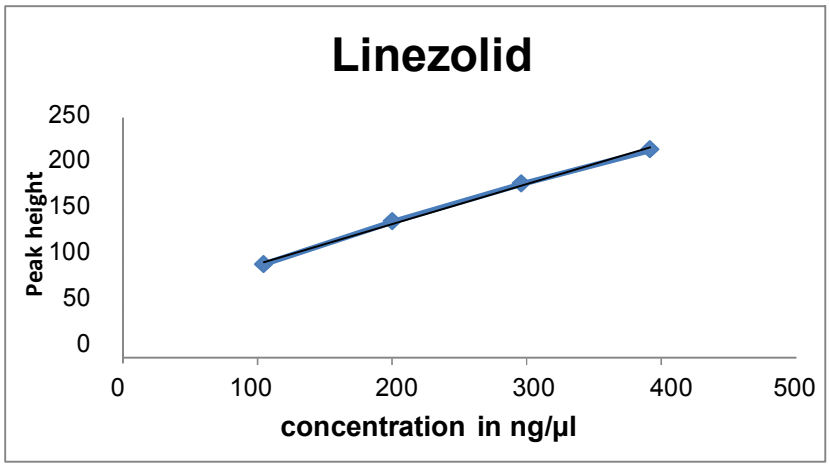


Figure 49: Calibration graph of Linezolid [Concentration v/s Peak height]

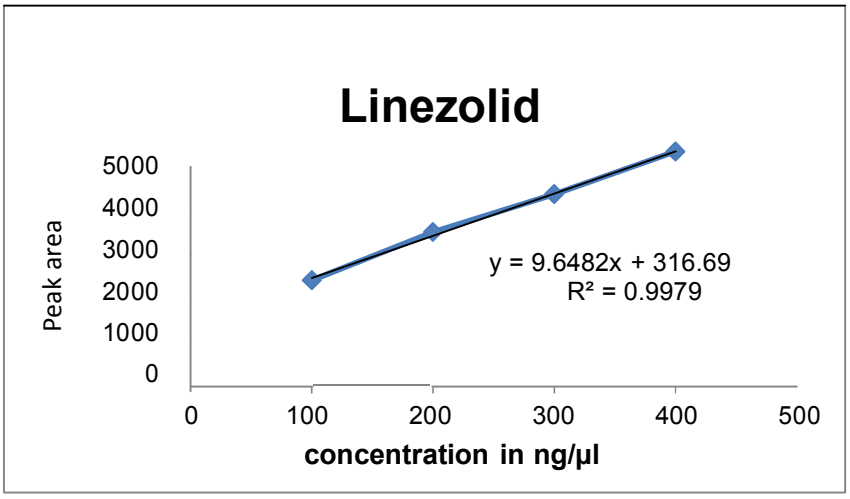


Figure 50: Calibration graph of Linezolid [Concentration v/s Peak area]

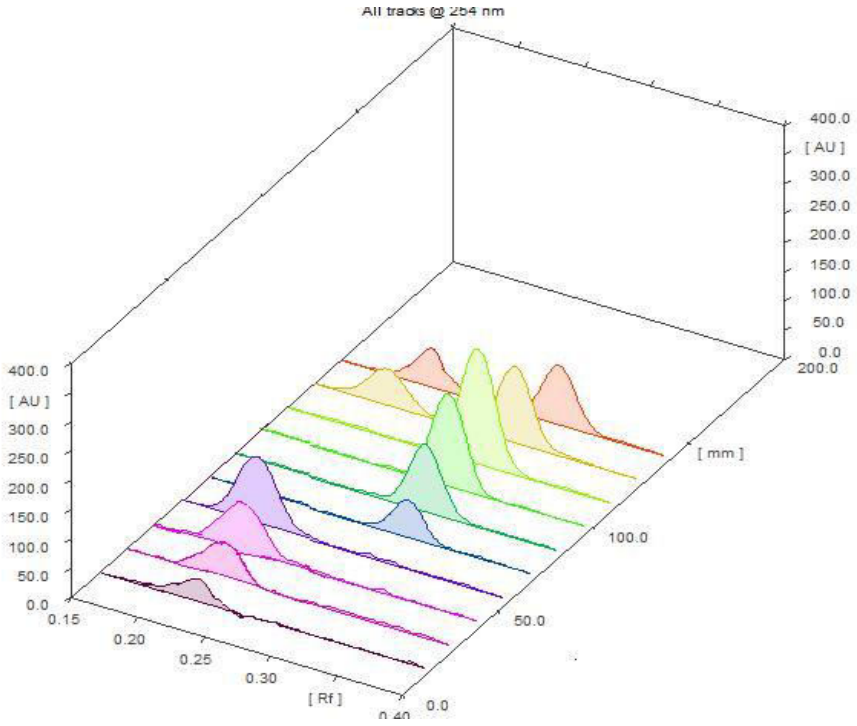


Figure 51: 3 D Overlay spectra of Cefuroxime axetil and Linezolid

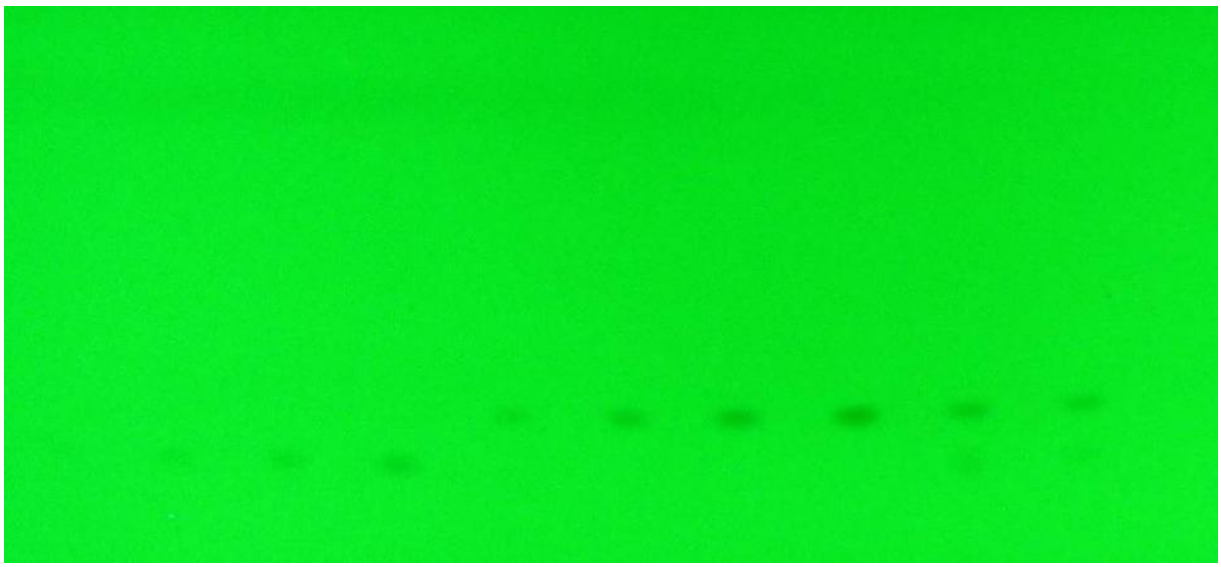
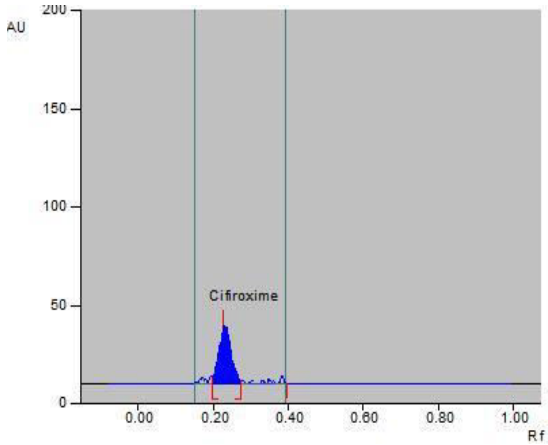
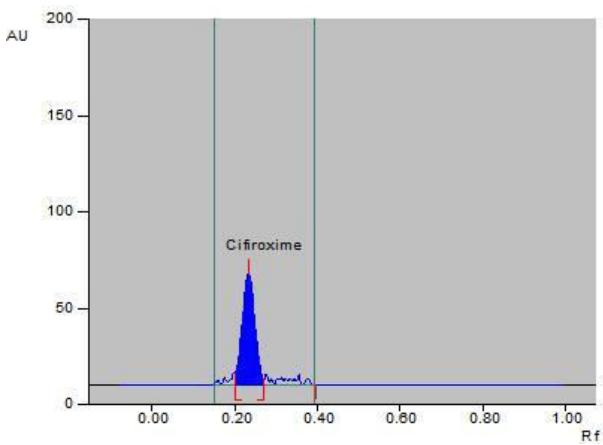


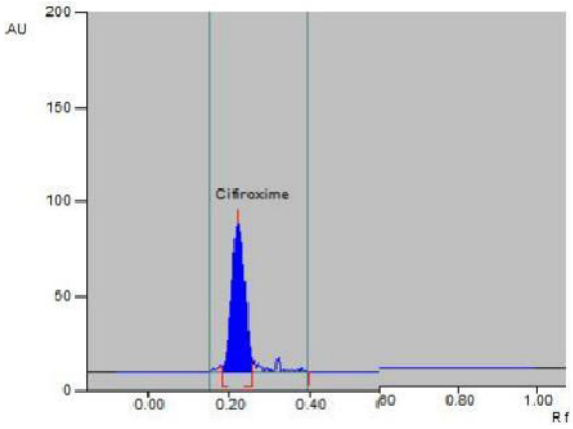
Figure 52 : Photograph of developed HPTLC plate



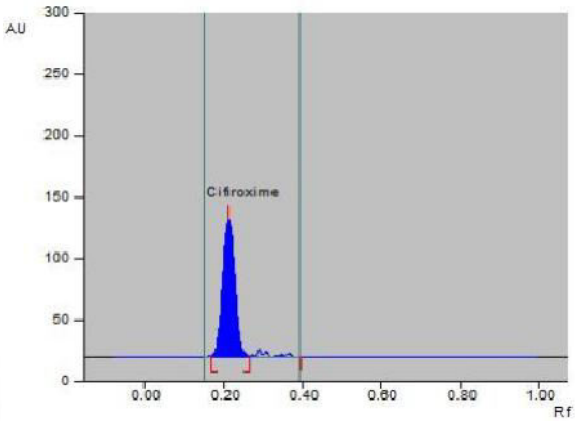
Cefuroxime axetil 100 ng/spot



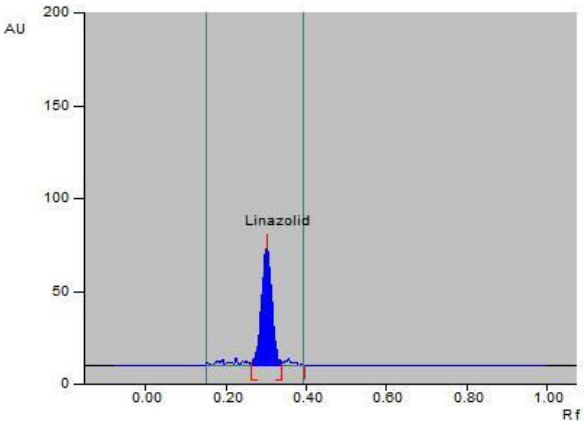
Cefuroxime axetil 200 ng/ spot



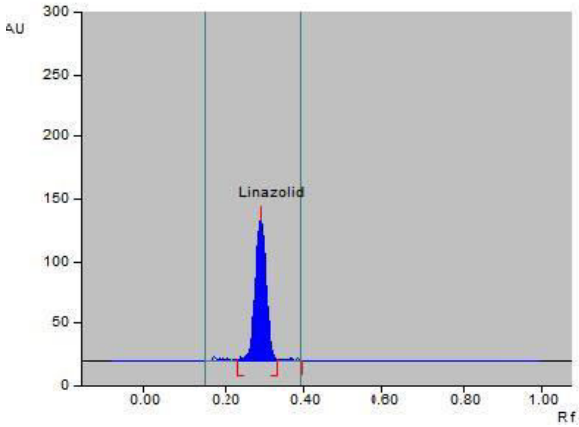
Cefuroxime axetil 300 ng/spot



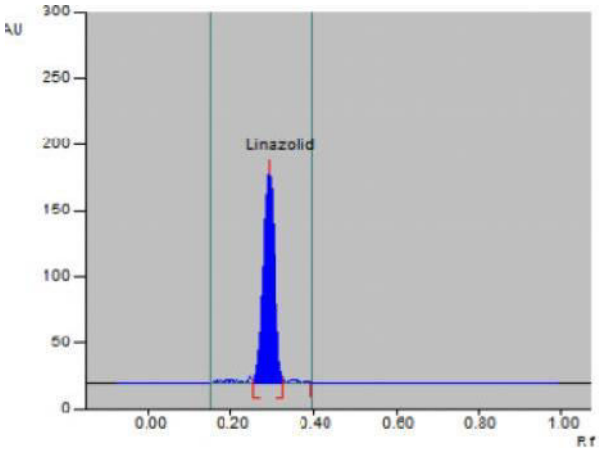
Cefuroxime axetil 400 ng/spot



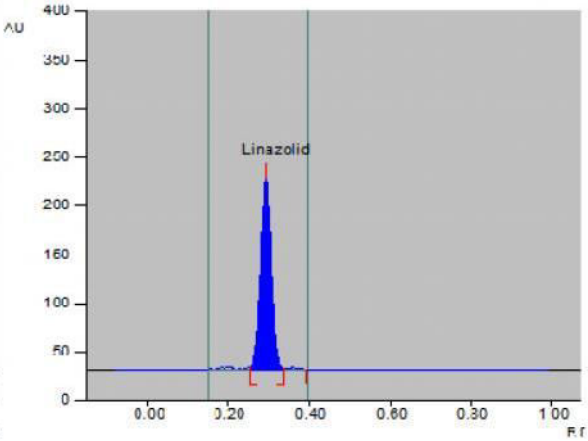
Linezolid 100 ng/spot



Linezolid 200 ng/ spot



Linezolid 300 ng/sopt



Linezolid 400 ng/spot

Figure 53 (a-h) HPTLC Chromatograms of CEF & LIN standard solutions

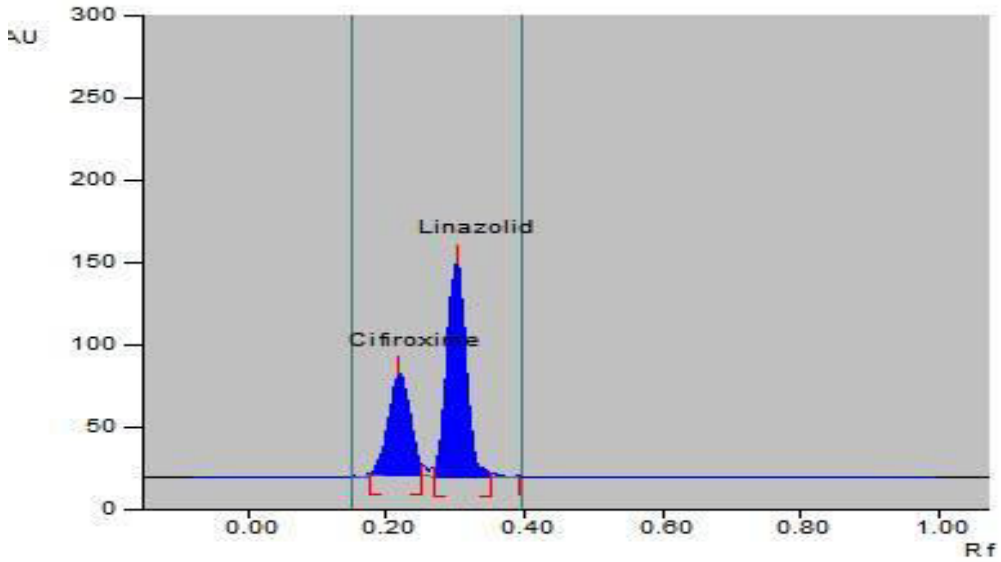


Figure 54: HPTLC chromatogram of standard drug mixture

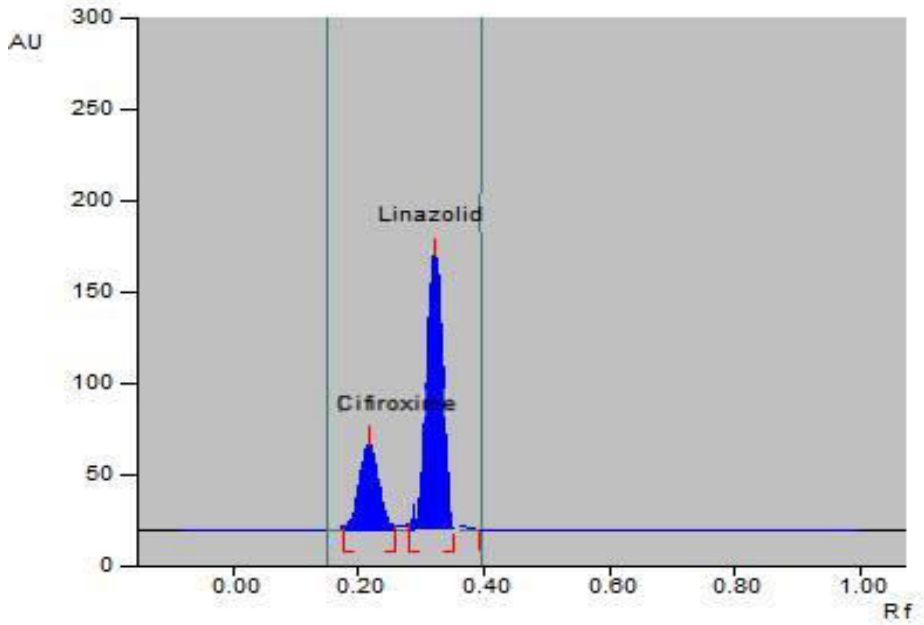


Figure 55: HPTLC chromatogram of sample Mixture

Results:

Each tablet contains (label claim), Cefuroxime axetil 500 mg Linezolid 600 mg

Average weight of one tablet 1.4826 g

Weight equivalent to 50 mg of Cefuroxime axetil 0.1482 g

Table 26: Average content per tablet determined by the proposed method

Drug	Height wise	Area wise
Cefuroxime axetil	496.81 mg	504.45 mg
Linezolid	596.35 mg	600.37 mg

Table 27: Percentage label claim

Drug	Height wise	Area wise
Cefuroxime axetil	99.32	100.89
Linezolid	99.33	100.06

Validation of proposed method

1. Accuracy

Accuracy of the proposed method was determined by recovery study. The recovery studies were performed by standard addition method at three concentrations (80%,100% and 120%) and percentage recovery was calculated.

- Twenty tablets of Oratil LZ (containing 500 mg Cefuroxime axetil and 600 mg Linezolid) were weighed and finely powdered in a glass mortar.
- A weight equivalent to 50 mg of Cefuroxime axetil was accurately weighed and trasferred to a stoppered flask .
- To this accurately weighed 40 mg Cefuroxime axetil and 48 mg of Linezolid were added (80%) and extracted with 25 ml of methanol initially by sonication for a period of 10 minutes.
- The solution was then trasferred to a 100 ml standard flask through a Whatman No.1 filter paper. The residue was further extracted twice, with 10 ml each of methanol and passed through the same filter paper and the volume was finally made up with methanol.
- From the above solution accurately pipetted out 1.0 ml solution and transferred in to a 10.0 ml standard flask. Then made up the solution to the mark.

- A chromatogram was developed using the above solution and scanned at 254 nm. The peak height and area were measured in three replicates and the amount recovered was estimated.
- In a similar way recovery studies for 100% and 120% were conducted and peak height and peak area were measured in three replicates for each level. The results and the statistically validated data are shown in the Table 27 to 31.

Table 28: Recovery results – Peak Height

Sl no	% recovery	Cefuroxime axetil		Linezolid	
		Peak Height	Peak Area	Peak Height	Peak Area
1	80	52.36	1234.56	118.68	2404.56
2		52.53	1234.11	118.66	2405.33
3		52.31	1234.17	118.67	2404.46
1	100	57.92	1366.15	129.24	2633.80
2		57.88	1365.31	129.27	2634.09
3		57.92	1365.57	129.25	2633.31
1	120	63.34	1492.63	139.82	2866.70
2		63.27	1492.37	139.86	2867.38
3		63.31	1493.41	139.84	2867.77

Table 29: Recovery results – Cefuroxime axetil

Level of recovery %	Amount present (µg/ml)	Amount added (µg/ml)	Drug recovered (µg/ml)		Drug recovery (%)	
			Height wise	Area wise	Height wise	Area wise
80%	50	40	40.21	40.11	100.53	100.28
	50	40	40.14	40.05	100.35	100.20
	50	40	40.12	40.08	100.30	100.21
100%	50	50	50.34	50.31	100.69	100.62
	50	50	50.26	50.24	100.53	100.49
	50	50	50.35	50.26	100.71	100.53
120%	50	60	60.22	60.11	100.36	100.18
	50	60	60.24	60.09	100.41	100.15
	50	60	60.27	60.17	100.45	100.28

Table 30: Recovery study-Cefuroxime axetil – Statistical validation

% Recovery		Mean	Standard deviation	% RSD	Coefficient of variation
80	Hight wise	100.39	0.1209	0.0012	0.0001
	Area wise	100.23	0.4350	0.0043	0.0004
100	Hight wise	100.64	0.9861	0.0979	0.0009
	Area wise	100.54	0.6650	0.0661	0.0006
120	Hight wise	100.40	0.4522	0.0450	0.0004
	Area wise	100.20	0.6800	0.0678	0.0006

Table 31: Recovery results – Linezolid

Level Of Recovery %	Amount present t	Amount added	Drug recovered (µg/ml)		Drug recovery (%)	
			Height wise	Area wise	Height wise	Area wise
80%	60	48	48.35	48.2	100.73	100.41
	60	48	48.33	48.24	100.68	100.50
	60	48	48.34	48.19	100.71	100.40
100%	60	60	60.21	60.07	100.35	100.12
	60	60	60.25	60.09	100.41	100.15
	60	60	60.22	60.05	100.37	100.09
120%	60	72	72.09	72.15	100.13	100.20
	60	72	72.13	72.18	100.18	100.25
	60	72	72.11	72.20	100.16	100.28

Table 32:Recovery study Linezolid- Statistical Validation

% Recovery		Mean	Standard deviation	% RSD	Coefficient of variation
80	Hight wise	100.75	0.2512	0.0249	0.0002
	Area wise	100.43	0.5505	0.0548	0.0005
100	Hight wise	100.37	0.3059	0.0304	0.0003
	Area wise	100.12	0.3004	0.0300	0.0003
120	Hight wise	100.15	0.2521	0.0251	0.0002
	Area wise	100.24	0.4046	0.0403	0.0004

2.Precision

Precision was determined at two levels: Repeatability and Intermediate precison.

Procedure for determination of Repeatability:

The repeatability of the method was studied by using 100% test concentration. For this, chromatogram was developed using mixed standard solution containing 50 µg/ml of Cefuroxime axetil and 60 µg/ml of Linezolid. The peak area and peak height were scanned six times at 254 nm and the data is shown in the table 32. The statistical validation data is shown in table 33

Table 33: Results of repeatability study

Sl No	Cefuroxime axetil		Linezolid	
	Peak area	Peak height	Peak area	Peak height
1	722.56	30.11	1475.20	75.28
2	722.51	30.09	1475.22	75.28
3	722.47	30.12	1475.20	75.27
4	722.62	30.09	1475.21	75.28
5	722.54	30.11	1475.20	75.27
6	722.61	30.11	1475.20	75.28

Table 34: Repeatability Statistical validation data

Drug	Method	Mean	Standard deviation	% RSD	Coefficient of variation
Cefuroxime axetil	Height wise	30.10	0.0122	0.0407	0.0004
	Area wise	722.55	0.0577	0.0079	0.00007
Linezolid	Height wise	75.27	0.0051	0.0067	0.00006
	Area wise	1475.20	0.0083	0.0005	0.000005

Intermediate precision: Inter day precision

The inter day precision study was carried out by scanning the chromatogram three times for three days for three different concentrations.

Standard stock solution having a concentration of 1000 µg/ml of Cefuroxime axetil and Linezolid were prepared. From this solutions, dilutions having concentrations of 100 µg/ml, 150 µg/ml, 200 µg/ml of Cefuroxime and Linezolid were prepared. Chromatogram were develop using this standard solutions and scanned at 254 nm. Peak height and peak area were measured three times on three days for each concentration. The data is given in the table 34, 35 and the statistical validation data is shown in tables 36 and 37.

Table 35: Inter day precision Cefuroxime axetil- Results

Sl No	Concentration (ng/spot)	Day 1		Day 2		Day 3	
		Peak height	Peak Area	Peak height	Peak Area	Peak height	Peak Area
1	100	29.82	727.91	29.30	728.06	29.61	727.82
2		29.49	728.12	29.48	728.19	29.74	727.82
3		29.64	728.08	29.56	728.22	29.43	728.03
1	200	58.44	1340.38	57.92	1340.14	58.51	1340.12
2		58.60	1340.26	58.28	1339.88	58.34	1340.25
3		58.74	1340.51	58.15	1340.22	58.65	1339.76
1	300	85.28	2014.32	84.81	2014.23	85.13	2014.52
2		84.89	2014.68	84.86	2014.11	85.06	2014.39
3		84.92	2014.52	84.70	2014.28	84.91	2014.41

Table 36: Inter day precision Linezolid results

Sl No	Concentration (ng/spot)	Day 1		Day 2		Day 3	
		Peak height	Peak Area	Peak height	Peak Area	Peak height	Peak Area
1	100	63.98	1233.72	64.13	1233.81	64.23	1233.69
2		64.05	1233.66	64.20	1233.89	64.21	1233.74
3		63.90	1233.69	64.15	1233.74	63.95	1233.59
1	200	114.08	2328.32	113.90	2328.25	113.97	2328.17
2		113.92	2328.41	113.86	2328.39	114.06	2328.20
3		113.95	2328.22	114.04	2128.24	114.04	2328.26
1	300	152.25	3190.33	152.14	3190.29	152.25	3190.44
2		152.11	3190.29	152.35	3190.36	152.33	3190.48
3		152.19	3190.46	152.26	3190.41	152.19	3190.51

Table 37: Inter day precision Statistical validation- CEF

Concentration (ng/spot)	Day	Method	Mean	Standard Deviation	%RSD	Coefficent of variation
100	1	Height	29.65	0.1652	0.0557	0.0005
		Area	728.03	0.1115	0.0153	0.0001
	2	Height	29.45	0.1331	0.0451	0.0004
		Area	728.15	0.0850	0.0116	0.0001
	3	Height	29.59	0.1556	0.0525	0.0005
		Area	727.89	0.1212	0.0166	0.0001
200	1	Height	58.59	0.1501	0.2561	0.0025
		Area	1340.38	0.1250	0.0932	0.0009
	2	Height	58.11	0.1823	0.3137	0.0031
		Area	1340.08	0.1777	0.0132	0.0001
	3	Height	58.50	0.1552	0.2652	0.0026
		Area	1340.04	0.2538	0.0189	0.0001
300	1	Height	85.03	0.2170	0.2552	0.0025
		Area	2014.53	0.2803	0.0139	0.0001
	2	Height	85.79	0.0818	0.0953	0.0009
		Area	2014.20	0.0873	0.0004	0.00004
	3	Height	85.03	0.1123	0.1307	0.0013
		Area	2014.44	0.0700	0.0034	0.00003

Table 38: Inter day precision LIN statistical validation

Concentratio n (ng/spot)	Day	Method	Mean	Standard Deviation	%RSD	Coefficent of variation
100	1	Height	63.97	0.0750	0.1172	0.0011
		Area	1233.69	0.0300	0.0024	0.00002
	2	Height	64.12	0.0360	0.0561	0.0005
		Area	1233.81	0.0750	0.0060	0.00006
	3	Height	63.95	0.1562	0.2442	0.0024
		Area	1233.67	0.0763	0.0061	0.00006
200	1	Height	113.98	0.0850	0.0745	0.0007
		Area	2328.31	0.0950	0.0040	0.00004
	2	Height	113.93	0.0945	0.0829	0.0008
		Area	2328.29	0.0838	0.0035	0.00003
	3	Height	114.02	0.0472	0.0413	0.0004
		Area	2328.21	0.0458	0.0019	0.00001
300	1	Height	152.18	0.0702	0.0461	0.0004
		Area	3190.36	0.08888	0.0027	0.00002
	2	Height	152.25	0.1053	0.0691	0.0006
		Area	3190.35	0.0602	0.0018	0.00001
	3	Height	152.25	0.0702	0.0461	0.0004
		Area	3190.47	0.0351	0.0011	0.00001

3. Linearity and range

The linearity study was conducted to evaluate the linear relationship across the range of analytical procedure. Linearity was determined using four different concentrations of each drug. Chromatogram was developed and peak area and peak height were determined by scanning at 254nm. Calibration graphs (concentration v/s peak area and concentration v/s peak height) were plotted for each drug and from this linearity was determined for each drug.The data showing the linearity of the developed method is furnished in Table 38.

Table 39 : Linearity and Range

Method parameters	CEF		LIN	
	Height wise	Area wise	Height wise	Area wise
Linearity range (ng/spot)	100-400	100-400	100-400	100-400
Slope	0.274	6.453	0.445	9.648
Intercept	2.855	71.47	22.185	316.69
R^2 value	0.9999	0.9997	0.9972	0.9979

4. **Limit of detection (LOD) amd limit of quantitation (LOQ).**

The LOD and LOQ were estimated from the set of 5 calibration curves used to determine the linearity of the developed method. Five calibration curves were drawn for each drug in their respective linearity range. From each calibration curve y-intercept and slope were substituted in the equation for finding LOD and LOQ.

LOD = 3.3 (σ/S) LOQ =10 (σ/S)

Where, σ = the standard deviation of y-intercepts of regression lines

S = the slope of calibration curve.

The data showing calibration LOD and LOQ are furnished in Table 39

Table 40 : Limit of detection and quantification

Drug	Method	Slope	Standard deviation	LOD ng/spot	LOQ ng/spot
Cefuroxime axetil	Height wise	0.274	1.5296	18.42	55.82
	Area wise	6.453	7.4327	3.80	11.51
Linezolid	Height wise	0.445	1.0647	7.89	23.92
	Area wise	9.647	10.0345	3.43	10.40

5. RESULTS AND DISCUSSION

5.1 RESULT AND DISCUSSION OF HPLC METHOD

Present report in this thesis is aimed at new analytical method development for the simultaneous estimation of Cefuroxime axetil and Linezolid by RP-HPLC method. From the literature review it was found that there was no single method for the simultaneous estimation of Cefuroxime axetil and Linezolid by RP-HPLC method. Hence new analytical method has been developed for the simultaneous estimation of Cefuroxime axetil and Linezolid by RP-HPLC method and validated according to ICH Q2B guidelines.

4.2.1. Selection of wavelength

100µg/mL solution of Cefuroxime axetil and 100µg/mL solution Linezolid was prepared using methanol as solvent. The above mentioned solutions were scanned individually from 190 to 400 nm in UV-Visible spectrophotometer. The optimal response for the overlain spectrum of Cefuroxime axetil and Linezolid was obtained at 284 nm. Hence the complete method was processed at the wavelength of 284nm.

Spectrums are shown inFig.No.9-11.

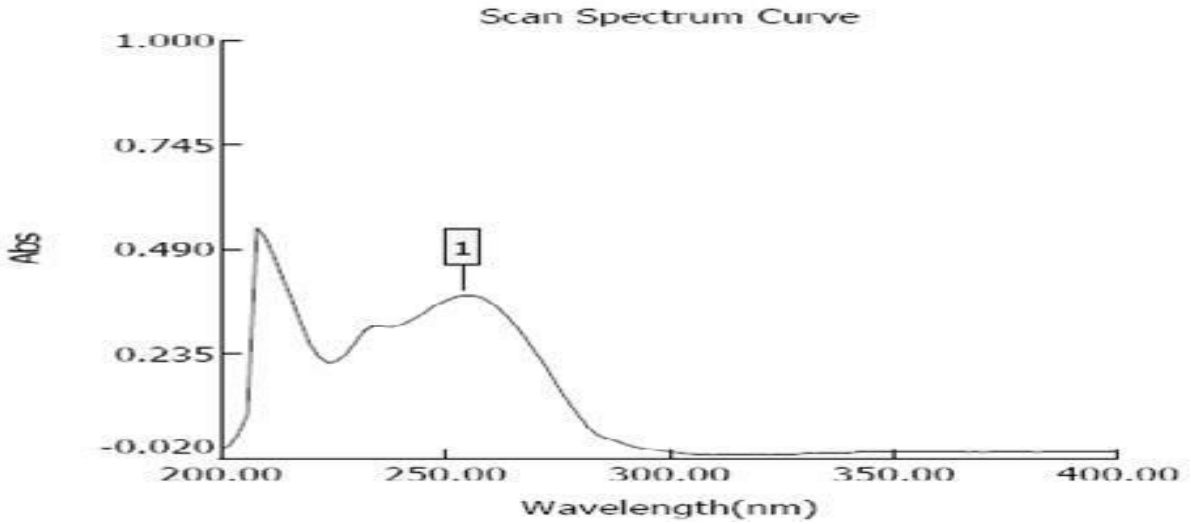


Fig.No.10: UV spectrum of Cefuroxime axetil

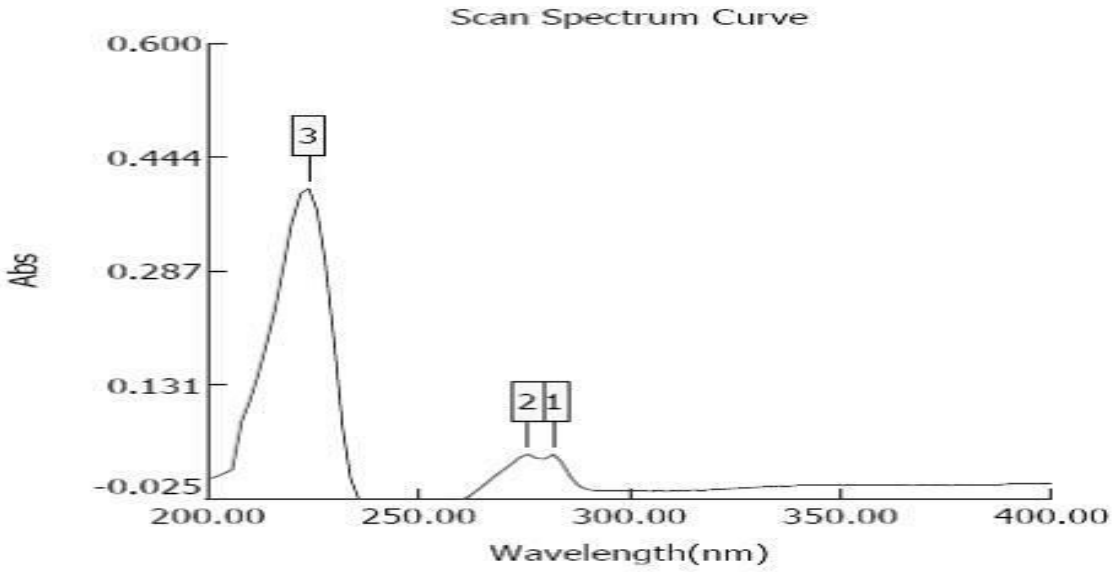


Fig.No.11: UV spectrum of Linezolid

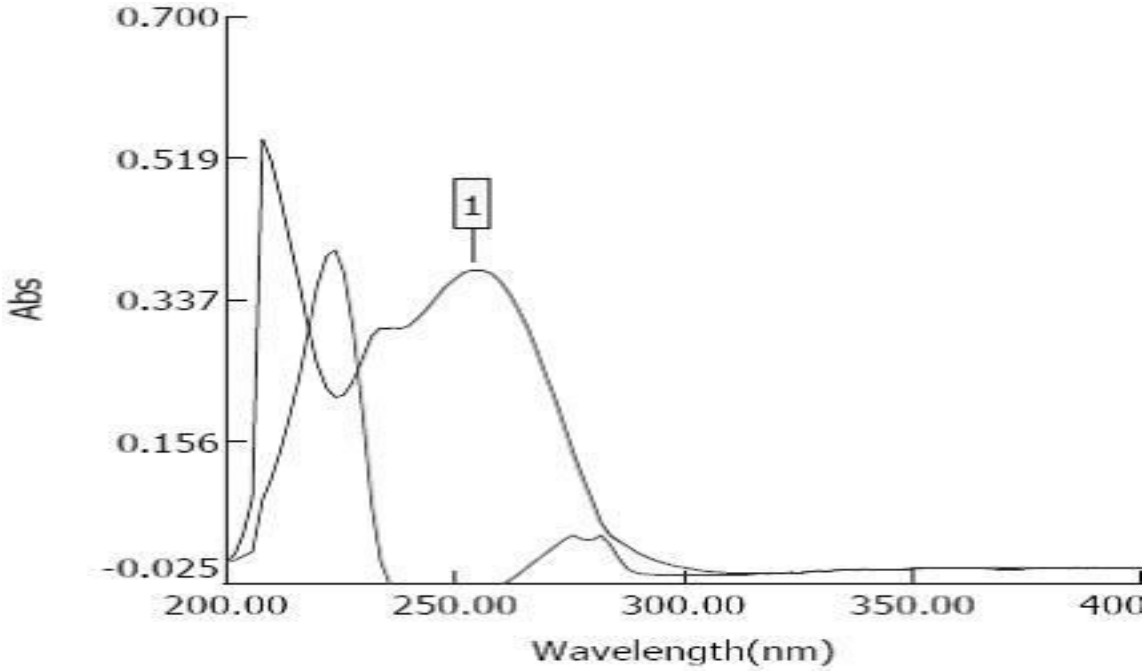


Fig.No.12: Overlay UVspectrum of Cefuroxime axetil and Linezolid

4.2.2 ANALYTICAL METHOD DEVELOPMENT

Several trials were made to get good peak resolution, acceptable plate count and tailing factor. Method was optimized for the simultaneous estimation of cefuroxime axetil and linezolid pharmaceutical dosage form.

OPTIMIZED METHOD

Mobile phase : phosphate buffer (pH2.8) : acetonitrile35:65v/v)

Diluent : Mobile phase was used as diluent.

Chromatographic conditions

Flow rate : 1 ml per min

Column : Symmetry Xterra C18 (4.6 x 150mm, 5μm) .

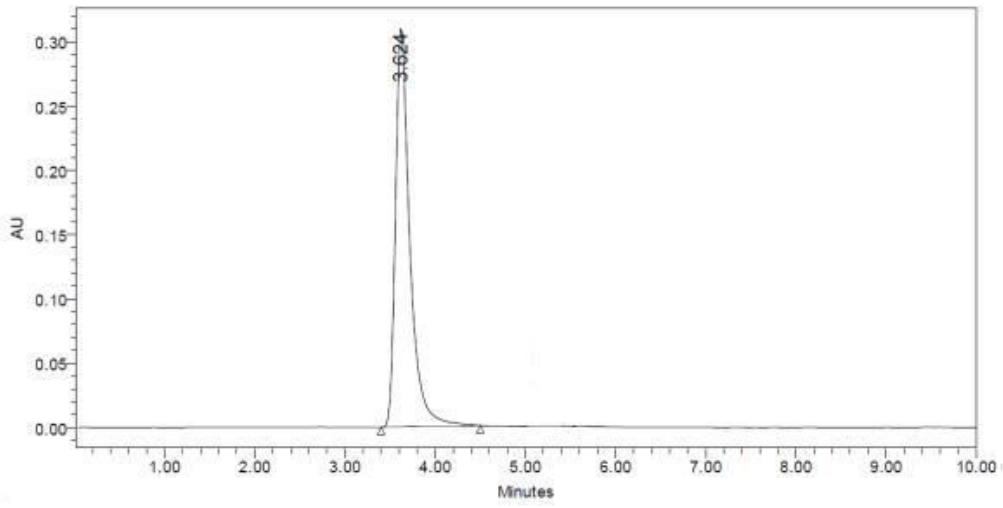
Detector wavelength : 284 nm

Column oven : Ambient

Injection volume : 20 μl

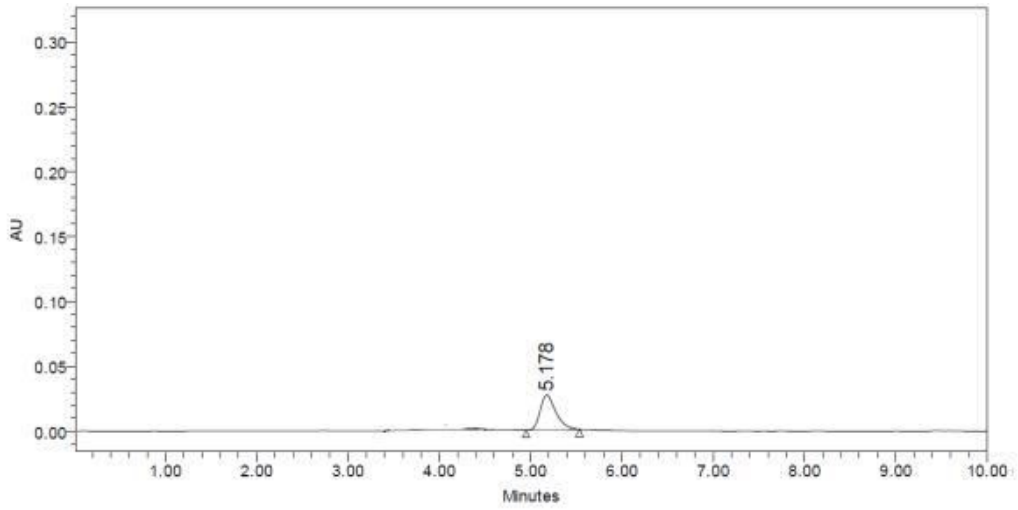
Run time : 10 min

Fig.No.13: Standard chromatogram for Cefuroxime axetil



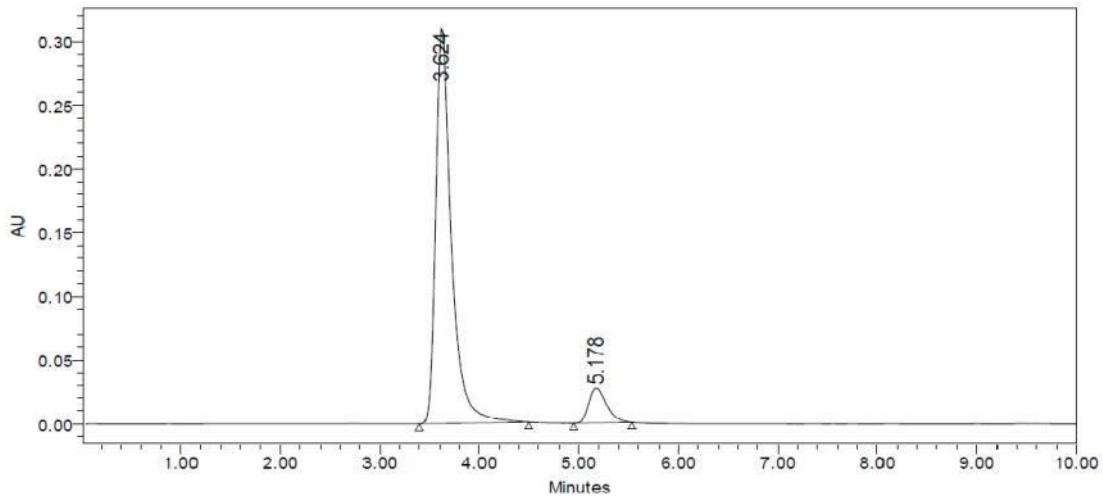
Name	Retention time	Area	USP Tailing	USP Plate count
CF	3.661	3463416	1.5	4890

Fig.No.14: Standard chromatogram for Linezolid



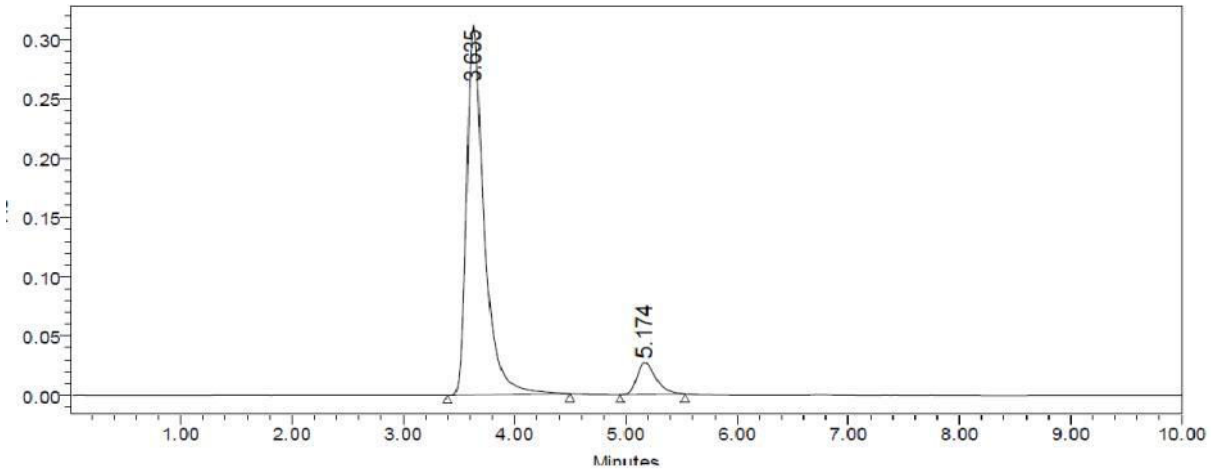
Name	Retention time	Area	USP Tailing	USP Plate count
LZ	5.116	324079	1.4	3586

Fig.No.15: Standard chromatogram for optimized method



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.661	3463416		1.5	4890
LZ	5.116	324079	5.1	1.4	3586

Fig.No.16: Sample Chromatogram for optimized method



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.642	3410176		1.5	4874
LZ	5.124	319570	5.1	1.4	3579

Fig.No.17: Blank chromatogram for optimized method

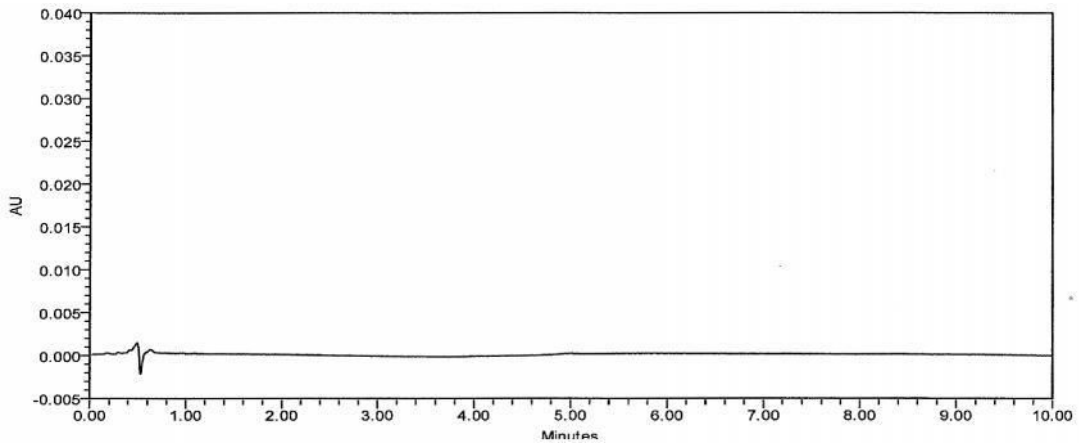


Table No.4: Results for standard and samples

S.No.	Name of the drug	Concentration	Area	Retention time
1.	Cefuroxime axetil standard	100µg/ml	3429046	3.624
2.	Linezolid standard	100µg/ml	320202	5.178
3.	Cefuroxime axetil and Linezolid standard solution	100µg/ml	3429046 and 320202	3.624 and 5.178
4.	Oractil-LZ tablet solution	100µg/ml	3402667 and 318846	3.635 and 5.174

The retention times for Cefuroxime axetil and Linezolid were found to be 3.624 and 5.178 respectively. Percentage purity of Cefuroxime axetil and Linezolid found to be 98.7% w/w and 98.8%w/w respectively. Resolution between two analytes is good. No peak asymmetry was observed. No other impurity interference was seen. All the results were found to be within the acceptance criteria. Hence the method was considered to be optimized. Results are given inTable No.5

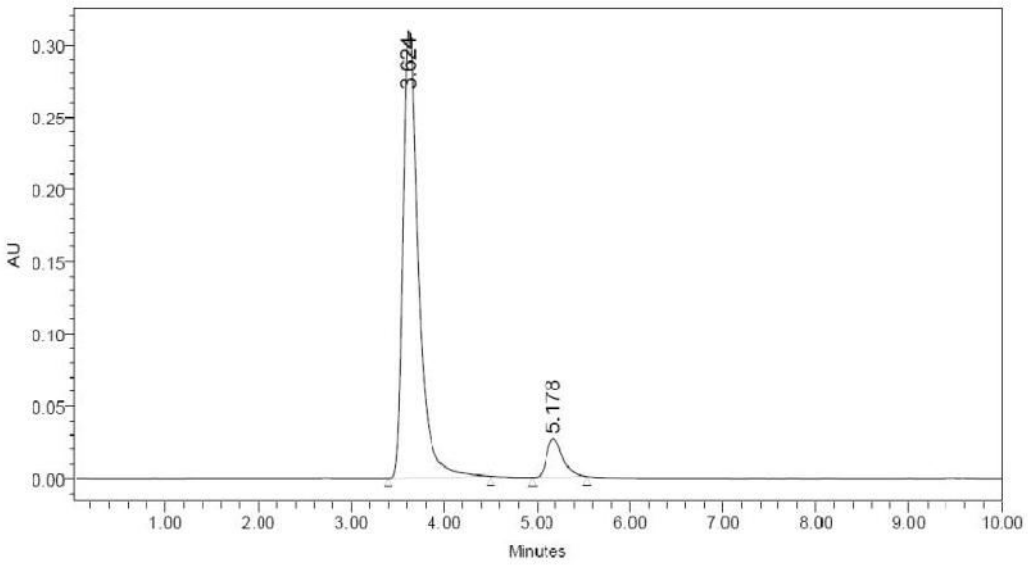
4.2.3. METHOD VALIDATION

1. SPECIFICITY

The chromatograms of standard and sample are identical with nearly same retention time. No interference due to placebo and sample at the retention time of analyte which shows that the method was specific. The chromatograms for specificity studies (standard, sample, placebo and blank) are represented asFig.No.17-20.

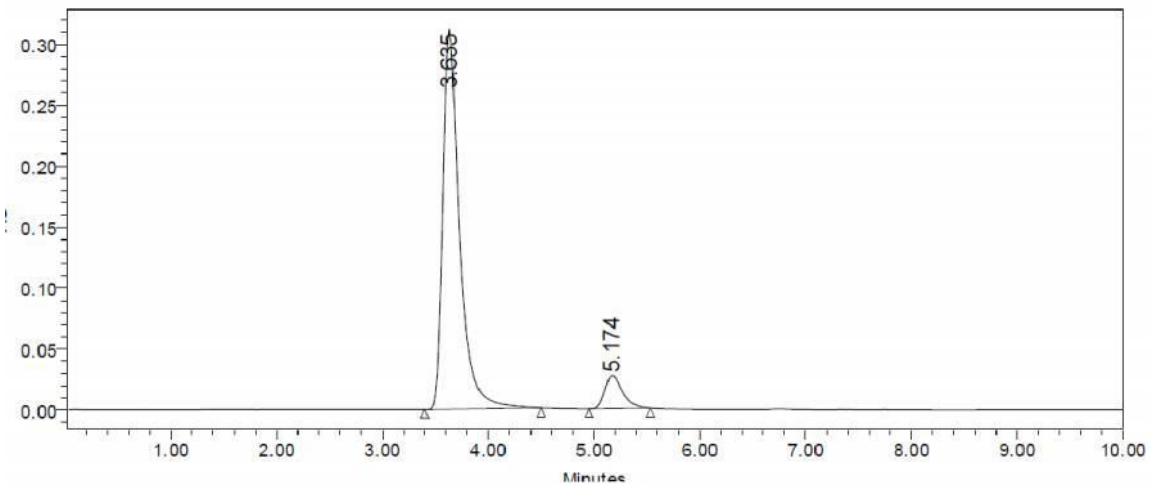
Fig.No.18:Standard chromatogram for Cefuroxime axetil and

Linezolid Identification



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.661	3463416		1.5	4890
LZ	5.116	324079	5.1	1.4	3586

Fig.No.19:Sample chromatogram for Cefuroxime axetil and Linezolid Identification



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.642	3410176		1.5	4874
LZ	5.124	319570	5.1	1.4	3579

Fig.No.20: Chromatogram for placebo interference

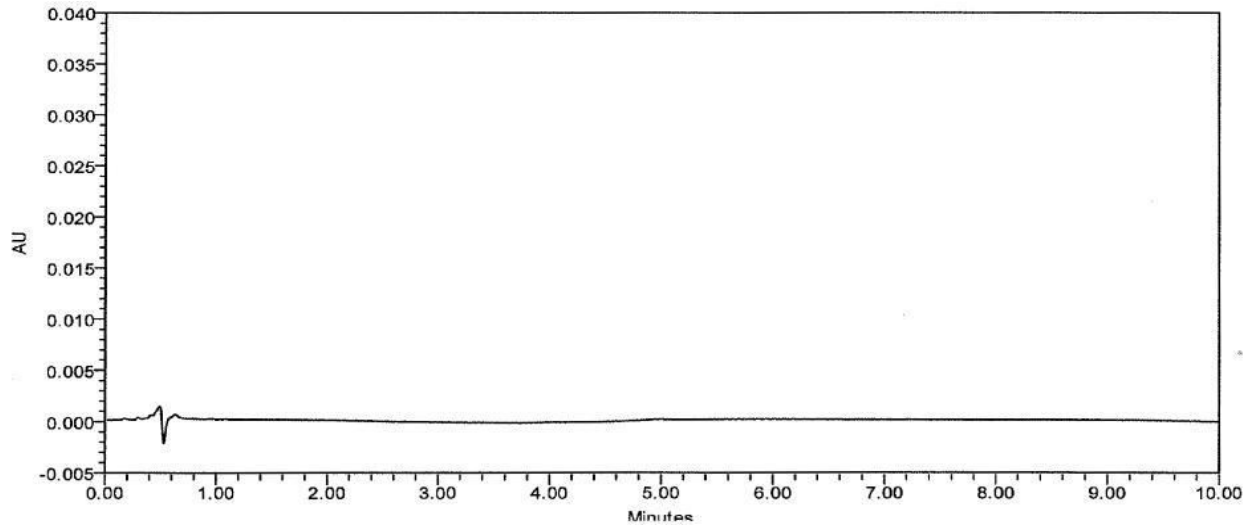
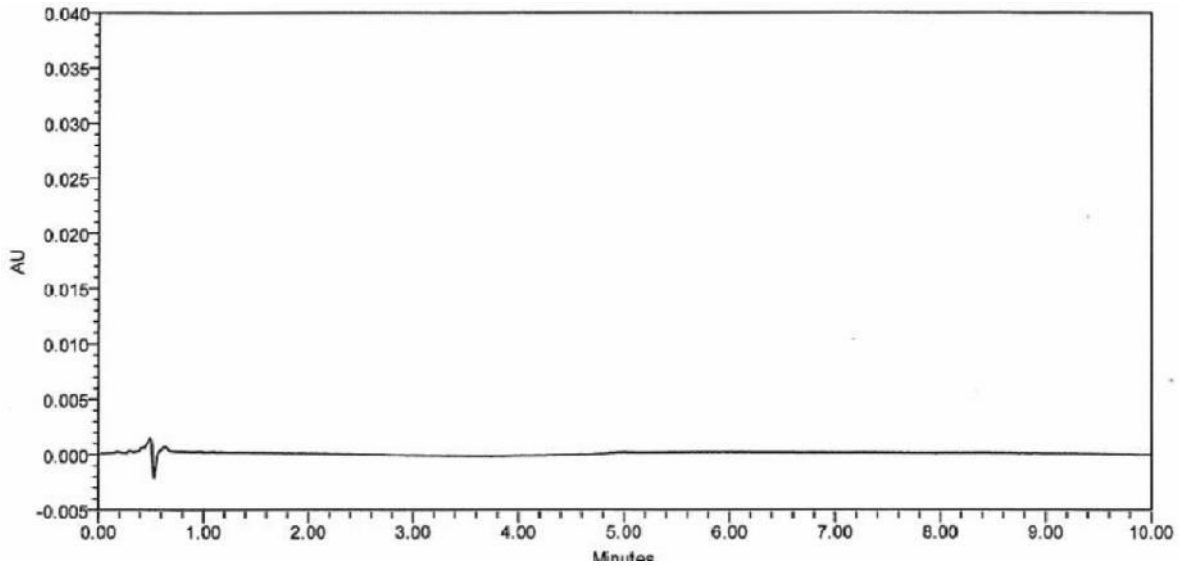


Fig.No.21: Chromatogram for blank interference

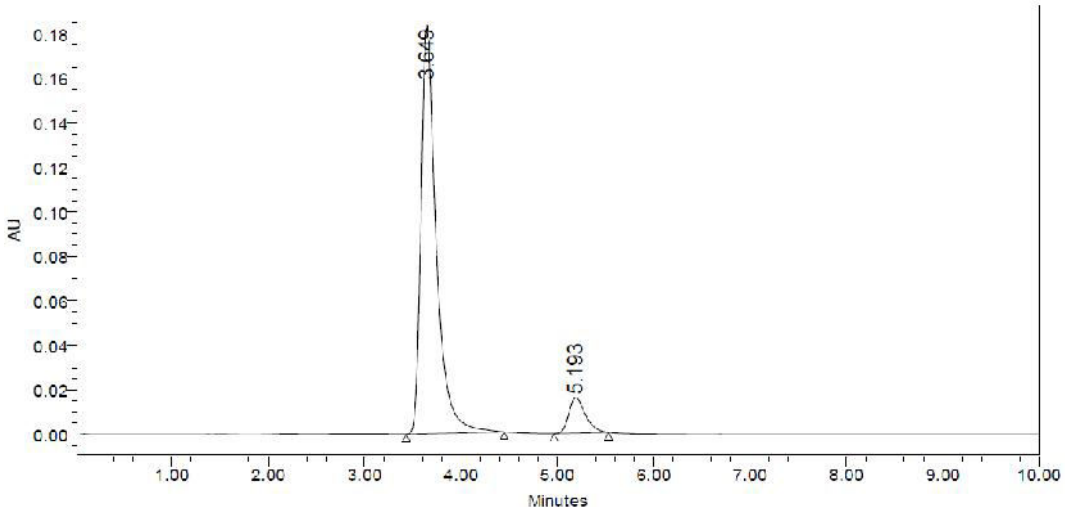


Chromatogram of standard and sample should be identical with near Retention time. Chromatogram of blank should not show any peak at the retention time of analyte peak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

2. LINEARITY

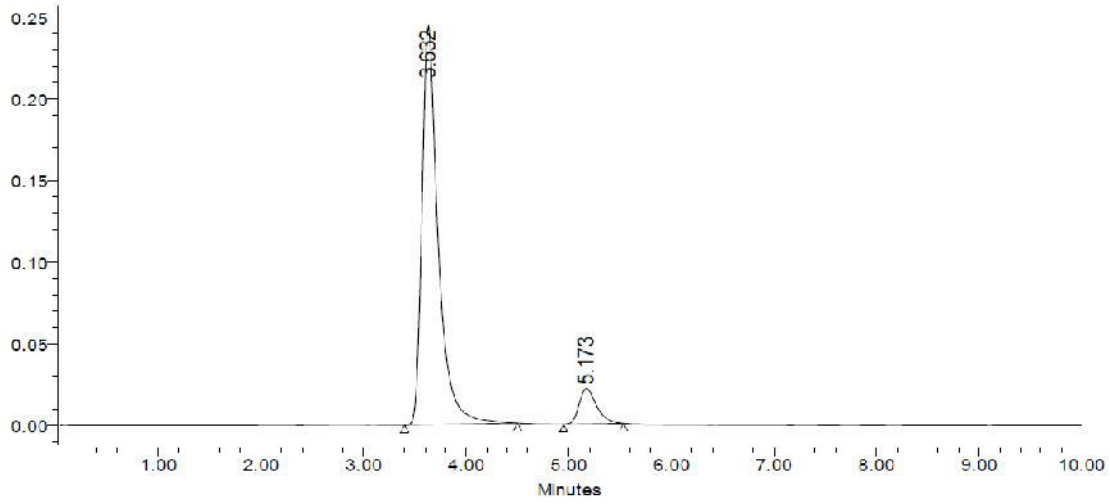
Linearity study was performed in the concentration range of 10-50 µg / ml. The Chromatograms for the linearity are shown in Fig.No.21-25. The linearity curve is plotted and shown in Fig.No.26 and 27. The data of linearity is tabulated in Table No.6and 7.

Fig.No.22. Chromatogram for Linearity 10 µg / ml



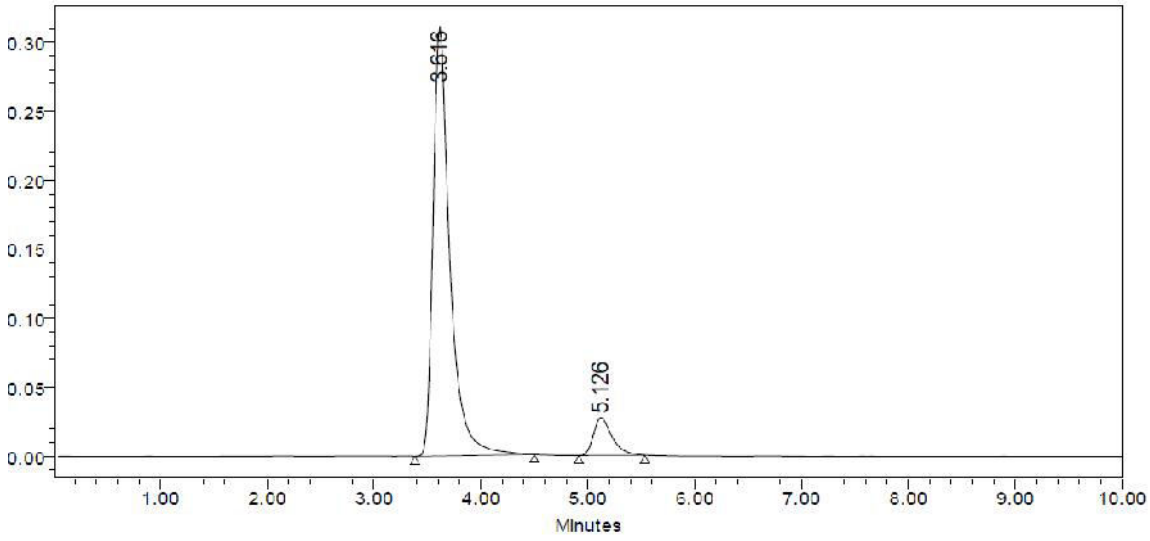
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.649	2011574		1.5	4993
LZ	5.193	189398	5.1	1.4	3489

Fig.No.23:Chromatogram for Linearity 20 µg / ml



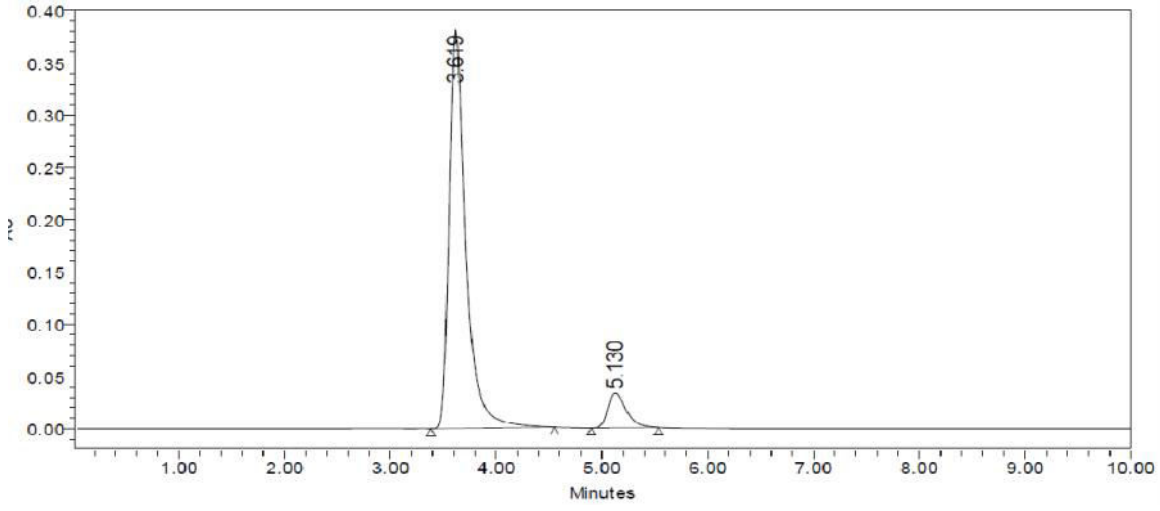
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.632	2681557		1.5	4864
LZ	5.173	258339	5.1	1.4	3902

Fig.No.24 Chromatogram for Linearity 30 µg / ml



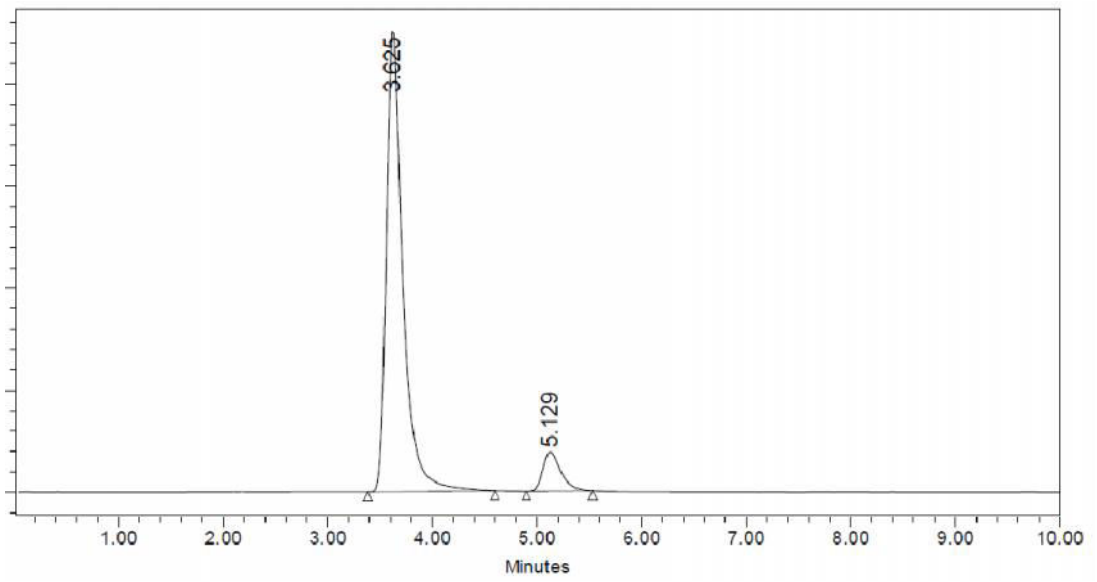
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.616	3390741		1.5	4740
LZ	5.126	321850	5.1	1.4	3237

Fig.No.25:Chromatogram for Linearity 40 µg / ml



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.619	4161134		1.5	4645
LZ	5.130	394694	5.1	1.4	3320

Fig.No.26:Chromatogram for Linearity 50 µg / ml



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.625	4964755		1.5	4874
LZ	5.129	459759	5.1	1.4	3579

CEFUROXIME AXETIL:

Fig.No.27:Calibration curve of Cefuroxime axetil

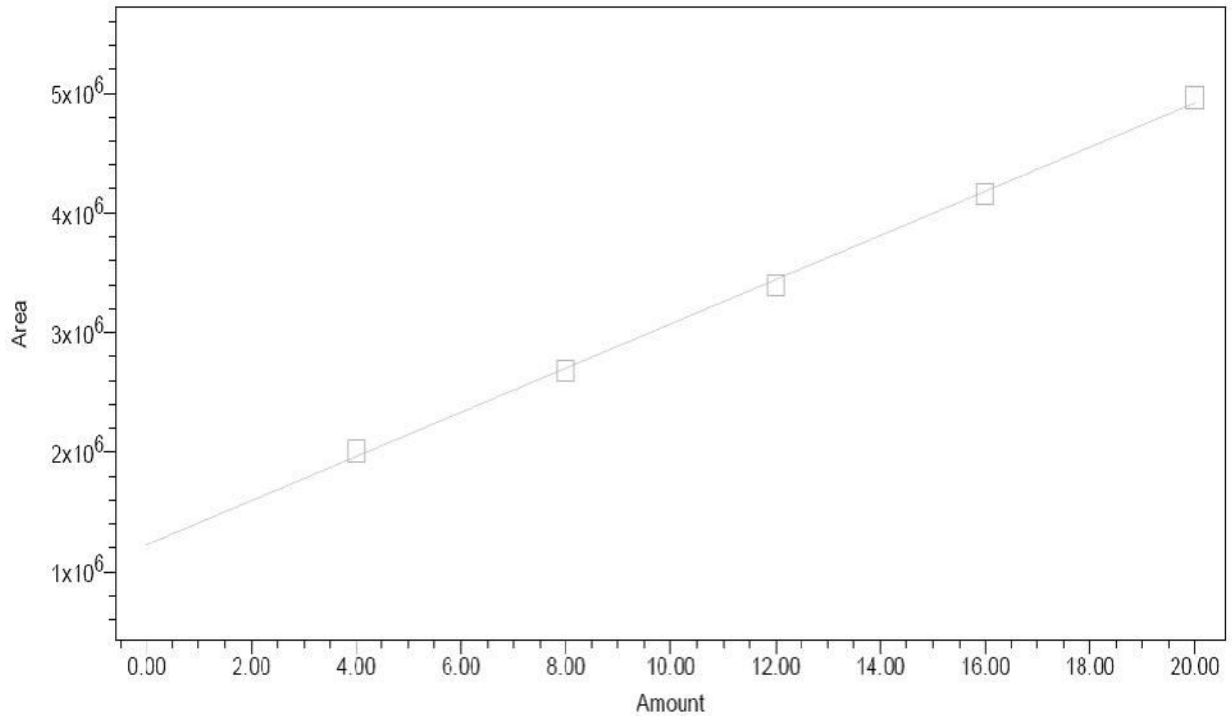


Table No.5: Linearity results for Cefuroxime axetil

S. No.	Linearity Level	Concentration	Area
1	I	4ppm	2011514
2	II	8ppm	2681557
3	III	12ppm	3390741
4	IV	16ppm	4161134
5	V	20ppm	4964755
Correlation Coefficient			**0.995**

Linezolid:

Fig.No.28:Calibration curve of linezolid

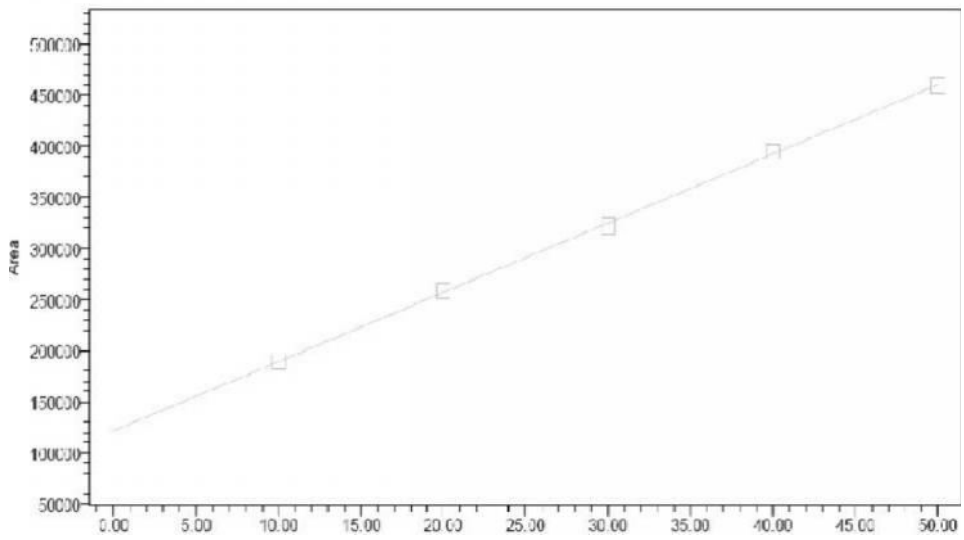


Table No.6: Linearity results for linezolid

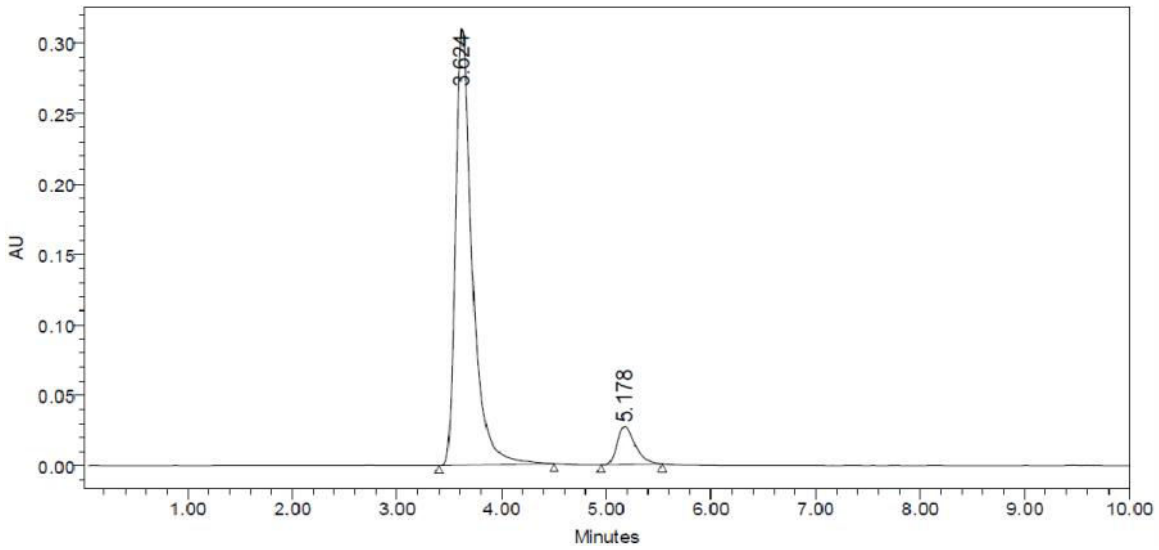
S. No.	Linearity Level	Concentration	Area
1	I	10ppm	189398
2	II	20ppm	258339
3	III	30ppm	321805
4	IV	40ppm	394694
5	V	50ppm	459759
Correlation Coefficient			**0.997**

Correlation co-efficient of cefuroxime axetil and linezolid was found to be 0.995 and 0.997 respectively (NMT 0.999).

3. ACCURACY

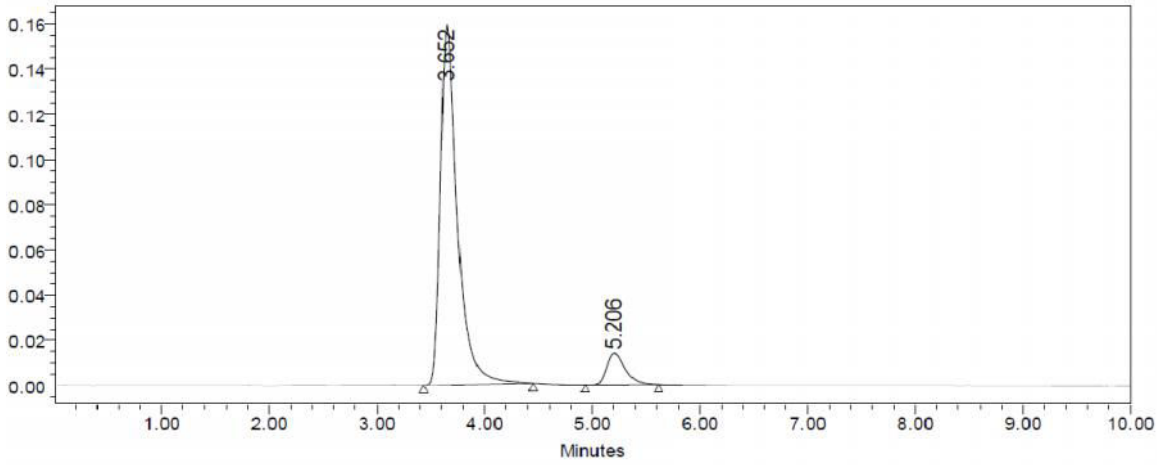
The percentage recoveries of pure drug from the analyzed solution of formulation are calculated in the recovery range from 50% to 150%. Standard and sample chromatograms for linearity are shown in Fig.No.28-31. The summary of accuracy results are tabulated in Table No.8 and 9.

Fig.No.29: Standard chromatogram for accuracy



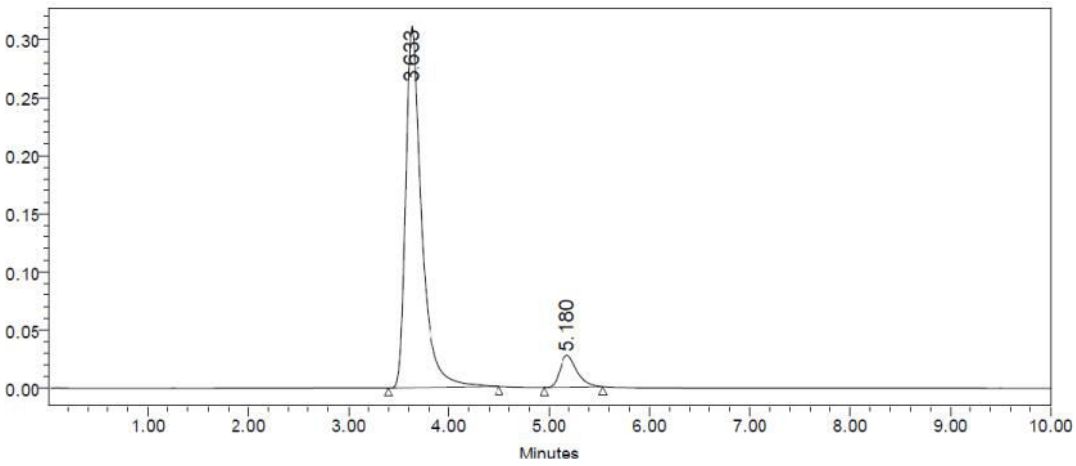
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.624	3429046		1.5	4864
LZ	5.178	320202	5.0	1.4	3469

Fig.No.30:Chromatogram of accuracy for 50 % Conc.



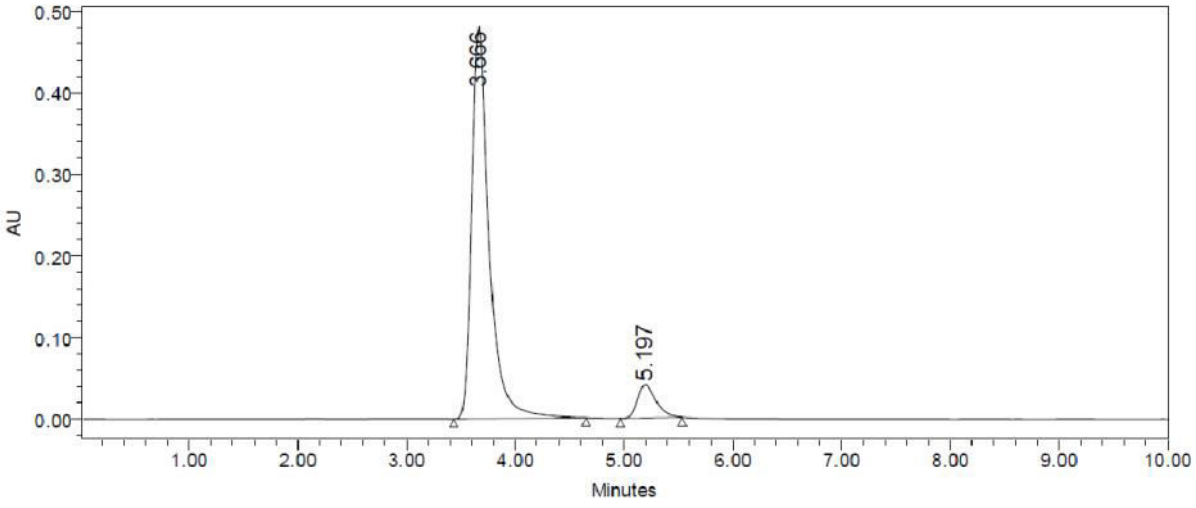
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.652	1742074		1.5	4874
LZ	5.206	163048	5.1	1.4	3579

Fig.No.31: Chromatogram of accuracy for 100 % Conc



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.633	3409693		1.5	4654
LZ	5.180	318680	5.1	1.4	3329

Fig.No.32: Chromatogram for accuracy for 150 % Conc.



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.666	5305423		1.5	4834
LZ	5.197	475301	5.1	1.4	3456

Table No.7: % Recovery results for CEFUROXIME AXETIL

Sample No.	Spike Level	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery
1	50 %	5	4.96	99.2%	100.3%
		5	4.99	99.8%	
		5	5.1	102%	
2	100 %	10	9.92	99.2%	99.4%
		10	9.94	99.4%	
		10	9.98	99.8%	
3	150 %	15.3	15.1	98.6%	99.3%
		15.3	15.2	99.3%	
		15.3	15.3	100%	

Table No.8: % Recovery results for LINEZOLID

Sample No.	Spike Level	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery
1	50 %	5	4.9	98%	100%
		5	5.1	102%	
		5	5	100%	
2	100 %	10	9.88	98.8%	99.13%
		10	9.91	99.1%	
		10	9.95	99.5%	
3	150 %	14.8	14.72	99.4%	99.69%
		14.8	14.79	99.9%	
		14.8	14.77	99.79%	

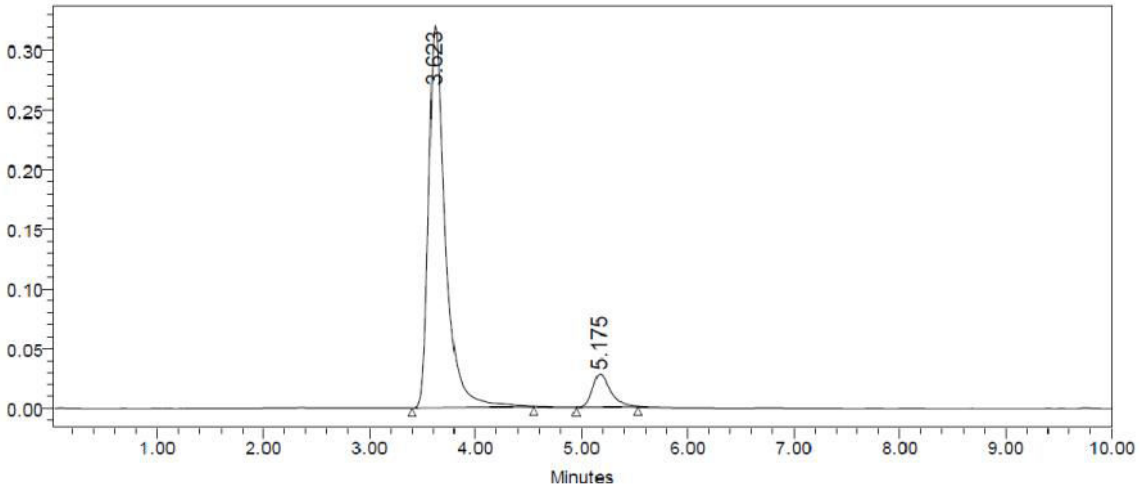
The % recovery for 50%, 100% and 150% accuracy level of cefuroxime axetil and linezolid was found to be within the range of 99.3-100.3% and 99.13-100% respectively (98.0 to 102.0%).

4. PRECISION

The RSD of % Recovery for cefuroxime axetil and linezolid chromatograms of repeatability precision and intermediate precision is calculated. It passes repeatability and intermediate precision. The results of precision are summarized in Table No.10-13.The Chromatograms related are represented as Fig.No.32 and 33.

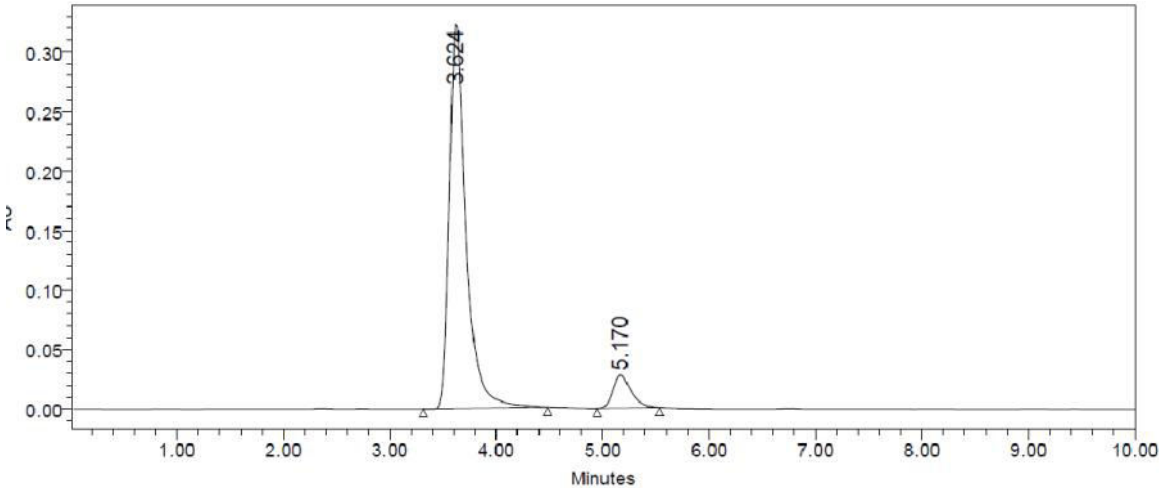
A) Repeatability

Fig.No.33.a): Sample chromatogram for repeatability



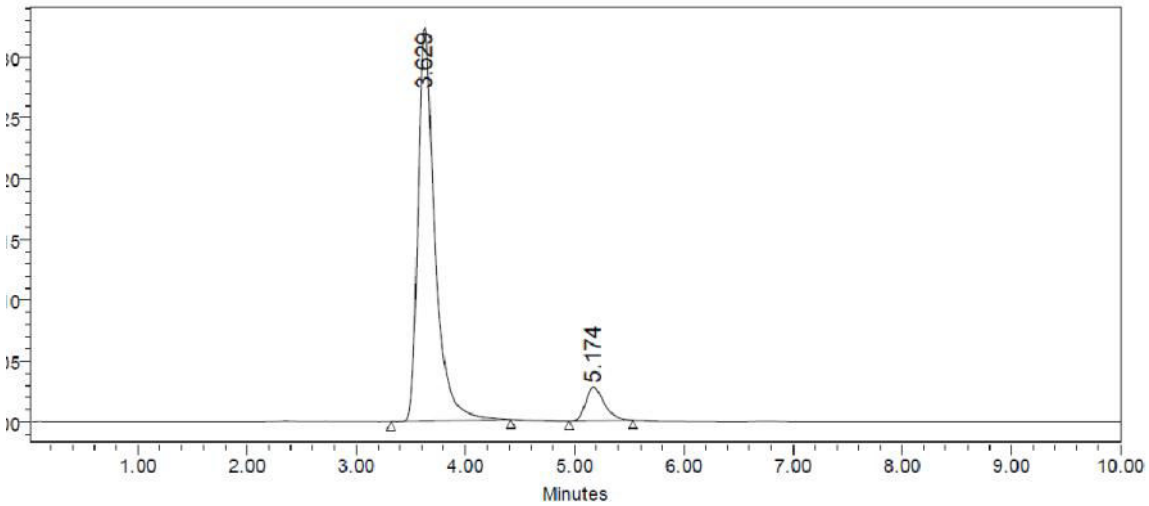
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.623	3480636		1.5	4874
LZ	5.175	323863	5.1	1.4	3579

Fig.No.33.b): Sample chromatogram for repeatability



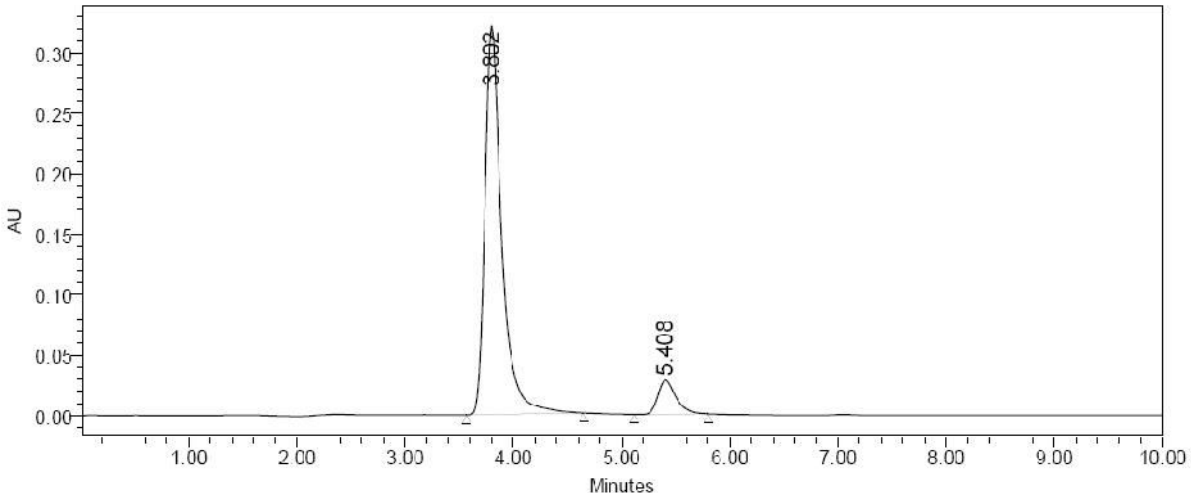
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.624	3463599		1.5	4874
LZ	5.170	325248	5.1	1.4	3579

Fig.No.33.c): Sample chromatogram for repeatability



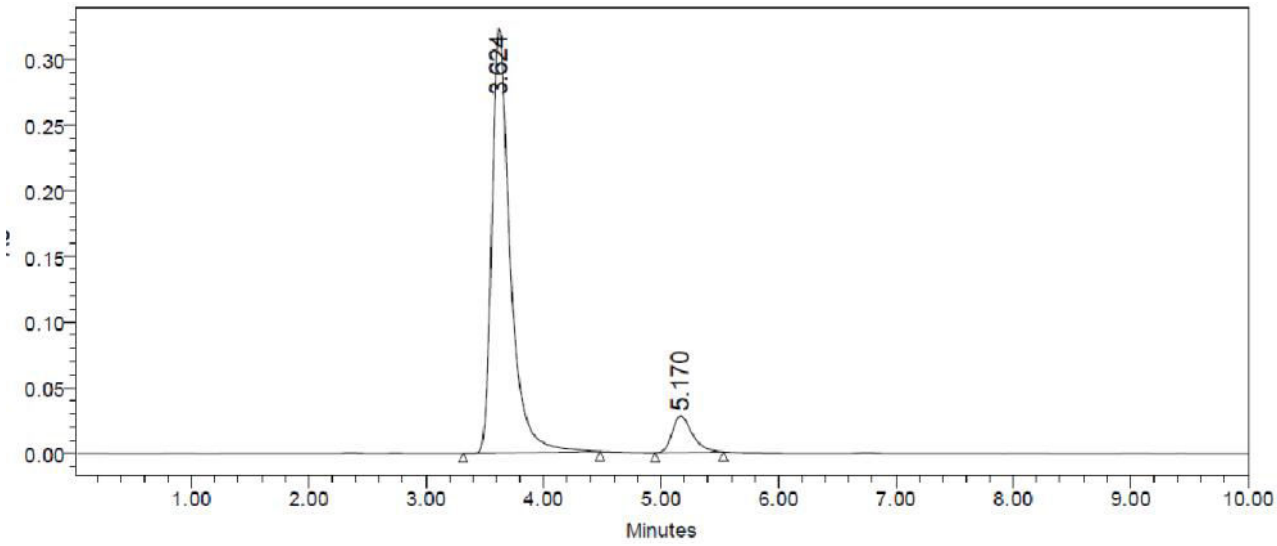
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.629	3498779		1.5	4574
LZ	5.174	322052	5.1	1.4	3339

Fig.No.33.d): Sample chromatogram for repeatability



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.629	3497870		1.5	4567
LZ	5.174	328133	5.1	1.4	3325

Fig.No.33.e): Sample Chromatogram for Repeatability



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.629	3490276		1.5	4500
LZ	5.174	328655	5.1	1.4	3325

Table No.9: Sample chromatogram values for repeatability of Cefuroxime axetil

Injection No	Peak area	% Recovery
1	3480636	99.4%
2	3463599	100%
3	3498779	99.0%
4	3497870	99.8%
5	3490276	99.2%
Mean	3486232	99.48%
SD	14601.3	0.415
% RSD	**0.42**	**0.42**

Table No.10: Sample chromatogram values for repeatability of Linezolid

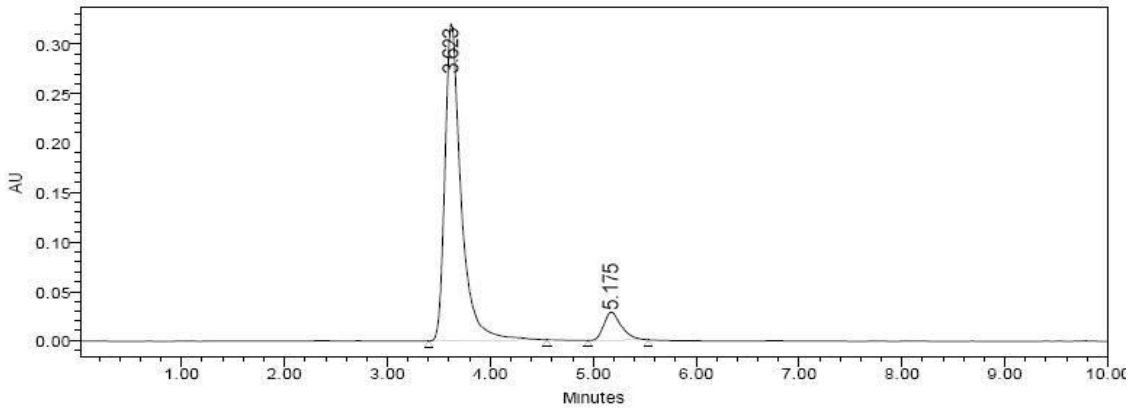
Injection No	Peak Area	% Recovery
1	323863	99.2%
2	325248	99.8%
3	322052	99.2%
4	328133	99.4%
5	328655	100%
Mean	325590	99.52
SD	2802.3	0.33
% RSD	**0.86**	**0.36**

The % RSD for area of five standard injections of repeatability of cefuroxime axetil and linezolid was found to be 0.42 and 0.36 respectively (NMT 2).

B. Intermediate precision (analyst to analyst variability):

Comparison of both the results obtained for two different analysts shows that the assay method was rugged for analyst-analyst variability. The chromatograms for intermediate precision are shown in Fig.No.34 and 35. The results of intermediate precision (Ruggedness) were found to be within the limits and are tabulated in Table No.12-15.

Fig.No.34: Chromatogram for intermediate precision (Day-1, Analyst-1)



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.623	3490276		1.5	4600
LZ	5.174	328655	5.1	1.4	3325

Table No.11: Intermediate precision results for Cefuroxime (Day-1, Analyst-1)

Parameter	Peak Area	% Assay
Avg*	3486743	99.10%
% RSD*	0.41	0.38

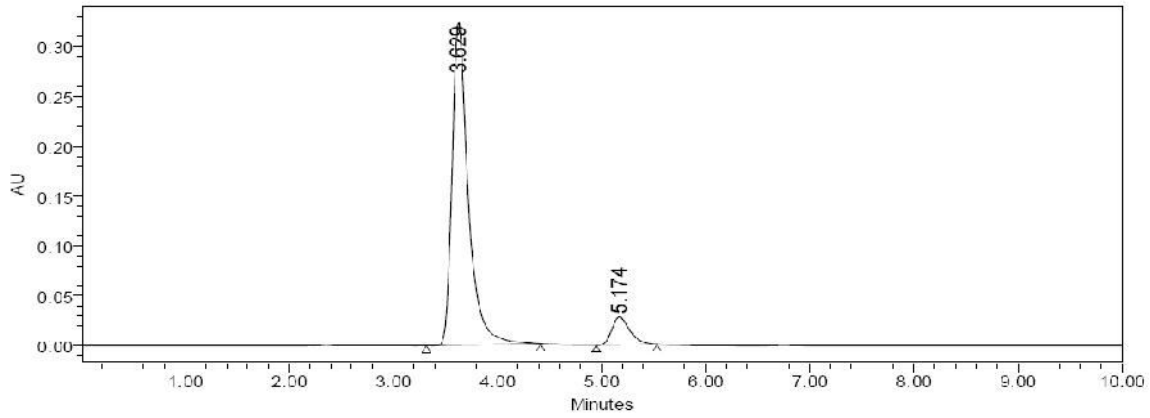
*Average of five determinations

Table No.12: Intermediate precision results for Linezolid Day-1, Analyst-1)

Parameter	Peak Area	% Assay
Avg*	3281662	99.98%
% RSD*	0.98	0.49

*Average of five determinations

Fig.No.35: Chromatogram for Intermediate precision (Day-2, Analyst-2)



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.629	3490276		1.5	4558
LZ	5.174	328655	5.1	1.4	3300

Table no.13: Intermediate precision results for Cefuroxime (Day-2, Analyst-2)

Parameter	Peak Area	% Assay
Avg*	3486232	99.48%
% RSD*	0.42	0.42

*Average of five determinations

Table no.14: Intermediate precision results for Linezolid (Day-2, Analyst-2)

Parameter	Peak Area	% Assay
Avg*	325590	99.52%
% RSD*	0.86	0.36

*Average of five determinations

The % RSD for the area of five standard injections for intermediate precision of cefuroxime axetil and linezolid was found to be 0.42 and 0.36 for day-1, analyst-1 and 0.43 and 0.26 for day-2, analyst-2 respectively (NMT 2).

8. LIMIT OF DETECTION (LOD)

The limit of detection was calculated from the linearity curve method using slope, and standard deviation of intercepts of calibration curve. Limit of Detection was found to be 0.003µg/ml for cefuroxime axetil and 0.09 µg/ml for linezolid The chromatograms are shown in Fig.No.41 and 42

For Cefuroxime axetil

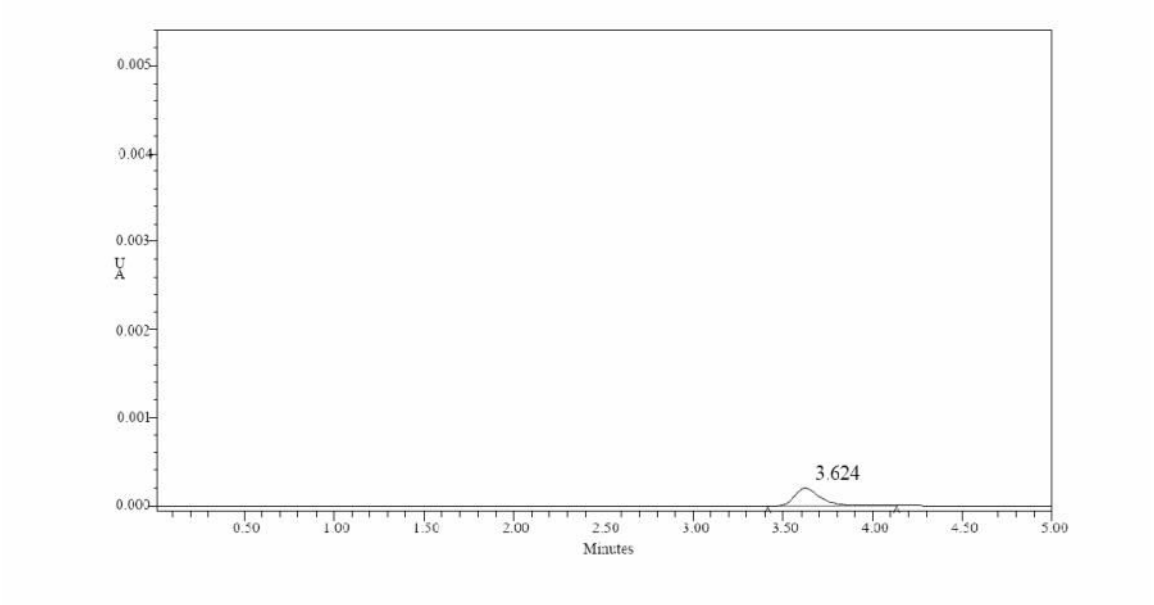


Fig.No.36: LOD chromatogram of Cefuroxime axetil

Calculation of S/N ratio

Average baseline noise obtained from blank : 52 µV

Signal obtained from LOD solution (0.25% of target assay concentration) : 154 µV

S/N = 154/52 = 2.96

For Linezolid

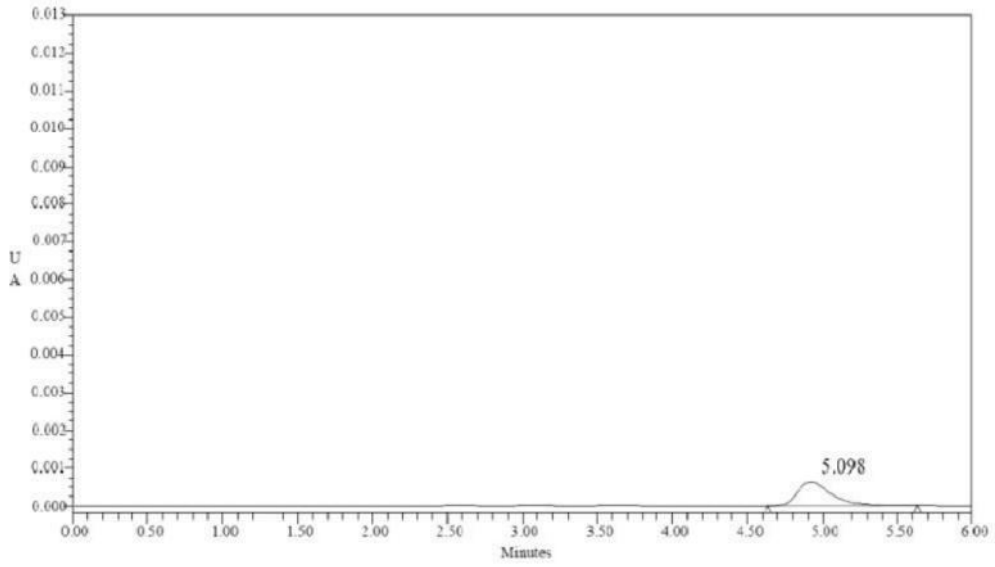


Fig.No.37: LOD chromatogram of Linezolid

Calculation of S/N ratio

Average baseline noise obtained from blank : 52 µV

Signal obtained from LOD solution (0.3% of target assay concentration) : 155 µV

S/N = 155/52 = 2.98

Limit of detection was found to be 2.96 for cefuroxime axetil and 2.98 for linezolid (NMT 3).

9. LIMIT OF QUANTIFICATION (LOQ)

The limit of quantification was calculated from the linearity curve method using slope, and standard deviation of intercepts of calibration curve. The chromatograms are shown in Fig.No.43 and 44

For Cefuroxime axetil

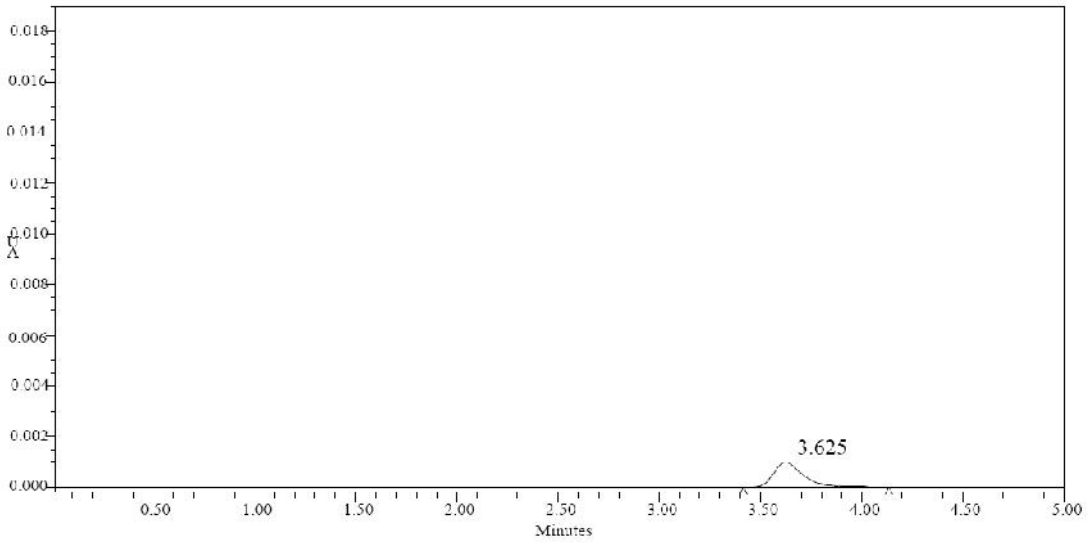


Fig.No.38: LOQ Chromatogram of Cefuroxime axetil

Calculation of S/N ratio

Average baseline noise obtained from blank : 52 µV

Signal obtained from LOQ solution (1% of target assay concentration) : 522µV

S/N = 522/52 = 10.

For Linezolid

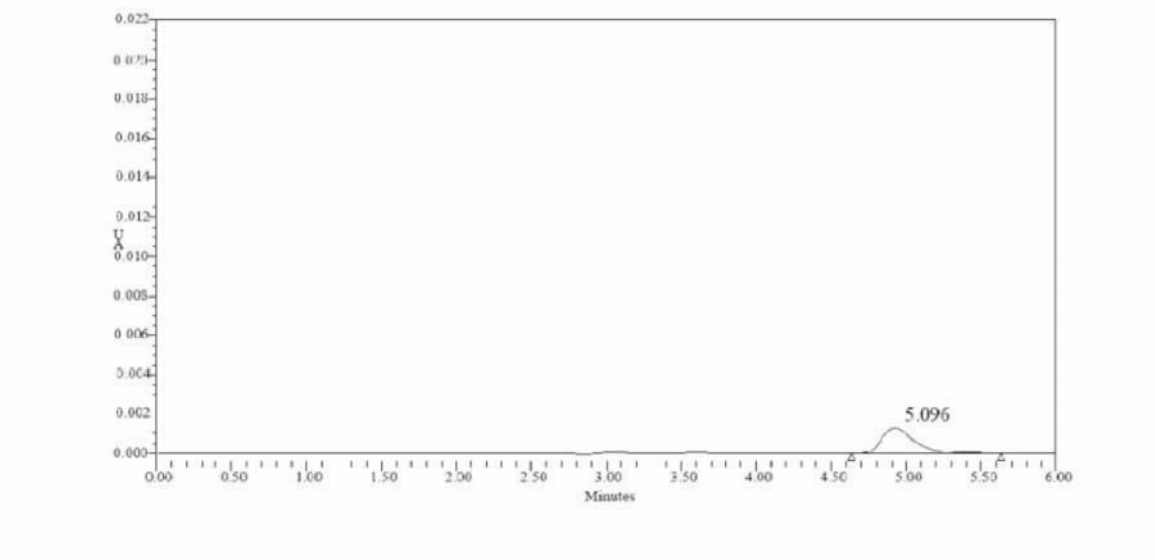


Fig.No.39:LOQ Chromatogram of Linezolid

Calculation of S/N Ratio

Average Baseline Noise obtained from Blank : 52 µV

Signal Obtained from LOQ solution (1.0% of target assay concentration) : 519µV

S/N = 519/52 = 9.98

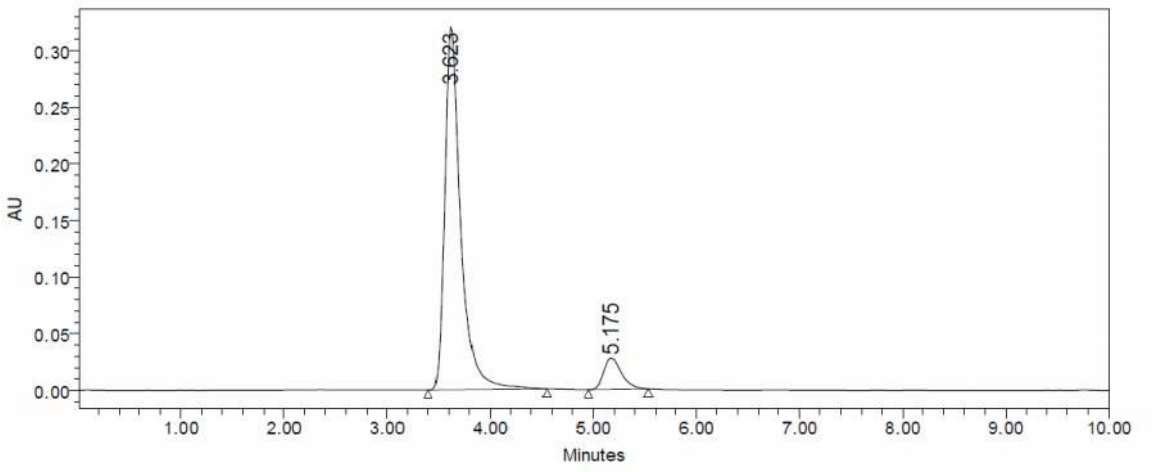
Limit of quantification was found to be 10 for cefuroxime axetil and 9.98 for linezolid NMT 10).

6. ROBUSTNESS

a) Effect of variation in flow rate

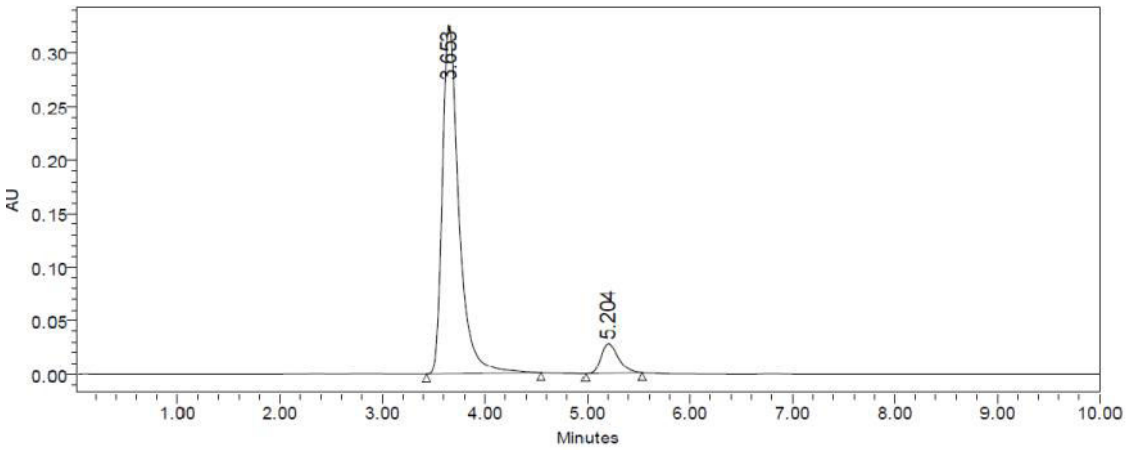
As the % RSD of retention time and asymmetry were within limits for variation in flow rate (± 0.1 ml). Hence the allowable flow rate should be within 0.4 ml to 0.6 ml. The chromatograms are recorded and shown in Fig.No.36 and 37.The results of robustness for effect of variation in flow rate are tabulated in Table No.16 and17.

Fig.No.40: Chromatogram for Robustness (flow rate-0.4 ml)



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.623	4051994		1.5	4800
LZ	5.175	395859	5.1	1.4	3525

Fig.No.41: Chromatogram for Robustness (flow rate-0.6 ml)



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.653	4964755		1.5	4235
LZ	5.204	459759	4.9	1.4	3179

Table No.15:Robustness results for Cefuroxime axetil

S.No	Flow rate (ml/min)	System suitability results	
		USP Plate count	SP Tailing
1	0.4	4859	1.62
2	0.5	4890	1.58
3	0.6	4895	1.58

***** Results for actual flow (0.5 ml/min) have been considered from assay standard

Table No.16: Robustness results For Linezolid

S.No	Flow rate (ml/min)	System suitability results	
		USP Plate count	USP Tailing
1	0.5	3330.4	1.52
2	0.7	3437.6	1.47
3	0.9	3228.7	1.47

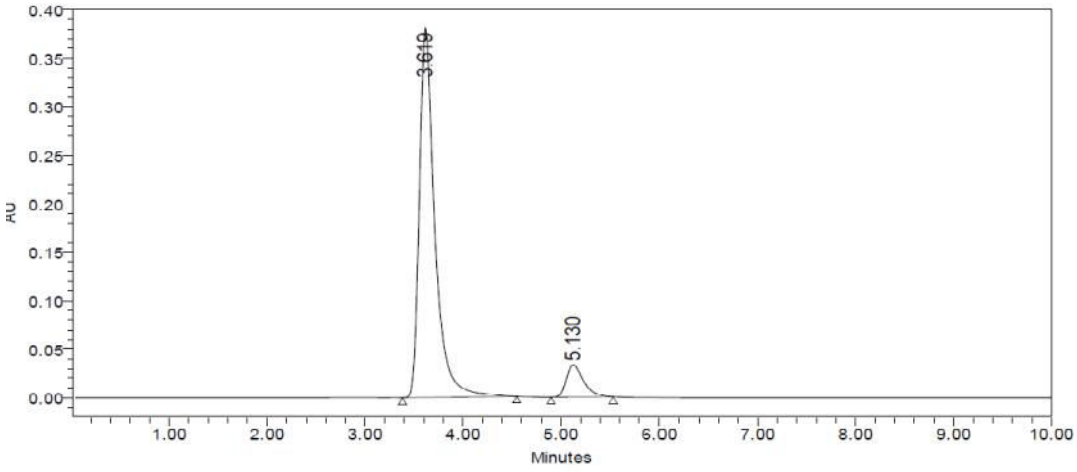
***** Results for actual flow (0.5 ml/min) have been considered from assay standard.

The % RSD of retention time and asymmetry were within limits for variation in flow rate (± 0.1 ml)

b) Effect of variation in mobile phase composition

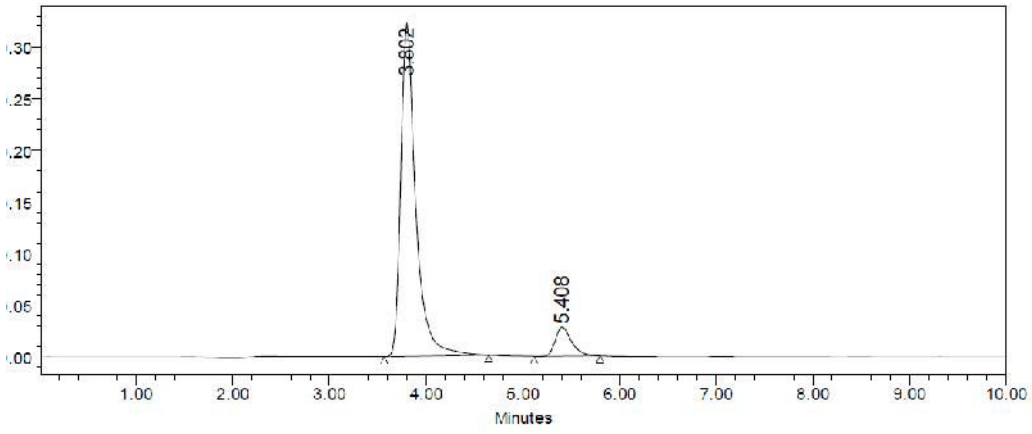
The chromatograms are shown inFig.No.38 and 39. The results of robustness for effect of variation in mobile phase composition are tabulated in Table No.18 and 19.

Fig.No.42: Chromatogram for Robustness (more organic)



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.619	3423569		1.5	6674
LZ	5.130	459759	4.8	1.4	4679

Fig.No.43: Chromatogram for Robustness (less organic)



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.802	4964755		1.5	6235
LZ	5.408	459759	5.1	1.4	4179

Table No.17: Robustness results for Cefuroxime axetil

S.No	Change in organic composition in the mobile phase	System suitability results	
		USP Plate count	USP Tailing
1	10% less	4899	1.52
2	*Actual	4857	1.52
3	10% more	4879	1.61

Table No.18: Robustness results for Linezolid

S.No	Change in organic composition in the mobile phase	System suitability results	
		USP Plate count	SP Tailing
1	10% less	3887	1.42
2	*Actual	3437	1.42
3	10% more	3985	1.51

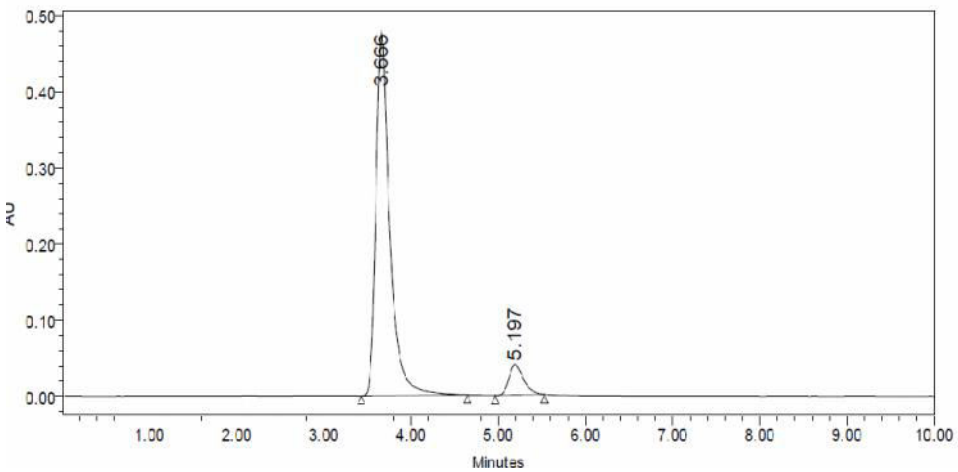
***** Results for actual mobile phase composition (65:35acetonitrile: phosphate buffer) has been considered from accuracy standard.

The % RSD of retention time and asymmetry were within limits for variation (± 2 %) in composition of mobile phase. Hence the method was found to be robust.

7. SYSTEM SUITABILITY: From the system suitability studies it was observed that % RSD of retention time was found to be 0.2, % RSD of peak area was found to be 0.2. Theoretical plates were found to be more than 3500. USP tailing factor was found to be 1.48 for cefuroxime axetil and 1.52 for linezolid All the parameters were within the limit. The chromatograms are shown in Fig.No.40. The results of system suitability studies are tabulated in Table No.20 and 21.

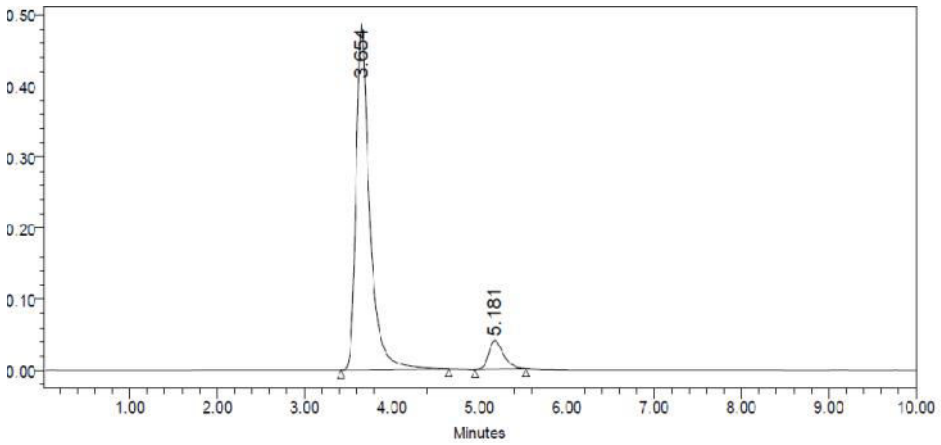
Chromatograms for System suitability:

Fig.No.44 a): Chromatograms for System suitability



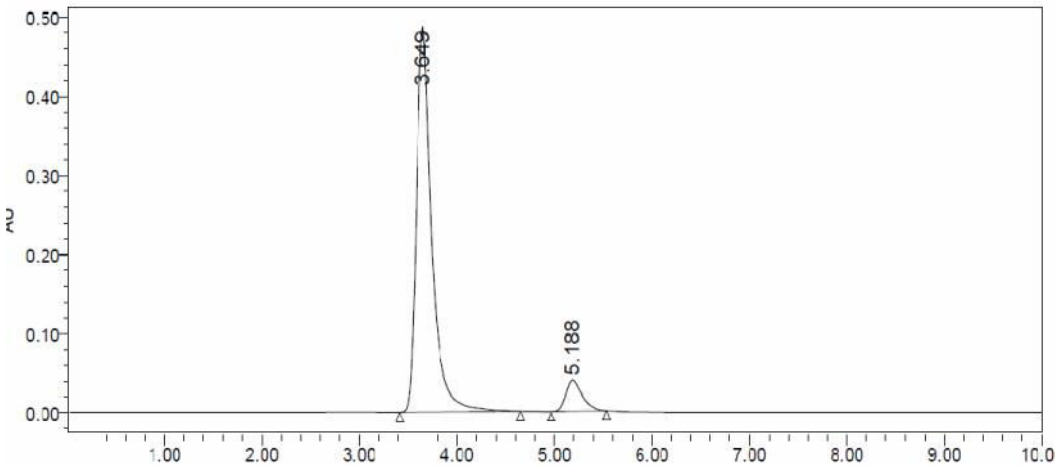
Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.666	5305432		1.6	4859
LZ	5.197	475301	5.1	1.5	3330

Fig.No.44 b): Chromatograms for System suitability



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.654	5318619		1.5	4890
LZ	5.181	479658	5.1	1.4	3330

Fig.No.44 c): Chromatograms for System suitability



Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
CF	3.666	5456432		1.6	6899
LZ	5.197	475301	5.1	1.5	4380

Table No.19: Chromatogram values for system suitability of Cefuroxime axetil

Injection	Retention time	Peak area	USP Plate count	USP Tailing
1	3.666	5305432	6859	1.62
2	3.654	5318619	6890	1.58
3	3.649	5319646	6998	1.58
Mean	3.656	5314566	6915.667	1.59333
SD	0.008	7926.638	72.96803	0.020394
% RSD	0.2389	0.1491	1.055	1.499

Table No.20: Chromatogram values for system suitability of Linezolid

Injection	Retention time	Peak area	USP Plate count	USP Tailing
1	5.197	475301	4330.4	1.52
2	5.181	479658	4337.6	1.47
3	5.188	476736	4228.7	1.47
Mean	5.188	477231.7	4298.9	1.486667
SD	0.008	2220.38	60.90148	0.028868
% RSD	0.154	0.4652	1.416	1.941

The overall summary of results for method validation parameters of cefuroxime axetil and linezolid are tabulated in Table No.22.

Table No.21: Table showing summary of results of method validation for Cefuroxime axetil and Linezolid

S.No	Parameter	Requirement	Results		Acceptance criteria
			CF	**LN**	
1.	Specificity	No interference	Pass	Pass	No interference
2.	Linearity	Correlation coefficient	0.9998	0.9997	NLT 0.999
3.	Accuracy	50% recovery	100.3%	100%	100 ± 2.0%
		100% recovery	99.4%	99.13%	
		150% recovery	99.3%	99.69%	
4.	Precision (repeatability)	%RSD	0.42	0.36	NMT 2%
5.	Intermediate precision	%RSD	0.03	0.89	NMT 1%
6.	Robustness	%RSD	0.43	0.36	NMT 1%
7.	System suitability	RT	3.654	5.181	-
8.		Tailing factor	1.6	1.4	NMT 2
9.		Plate count	4859	3330	NLT 3000
10.		Assay value	98.7%	98.8%	100 ± 2.0%

5.2 RESULT AND DISCUSSION-HPTLC

METHOD-II: HPTLC determination of Cefuroxime axetil and Linezolid in dosage form

- The solvent used for preparation of stock solution was methanol HPLC grade.
- The stationary phase was pre-coated plates of silica gel G 60 F60 F254 and the mobile phase used was Methanol: Toluene: Hexane (2:5:3, v/v/v).
- The R_f value was found to be 0.21 and 0.30for CEF and LIN respectively. The plate was scanned and quantified at 254 nm.
- Calibration curve for each drug was plotted using to parameters concentration v/s peak height. The linearity range of Cefuroxime axetiland Linezolidwere100-400ng/spot.
- The marketed product (Oratil LZ) containing 500 mg of Cefuroxime axetil and 600 mg of Linezolid was analyzed by the developed method and gave good results. Amount of drugs in analyzed dosage form was found to be
    - ❖ Cefuroxime axetil
      496.81mg by height wise

      504.45 mg by area wise
    - ❖ Linezolid
      596.35 mg by height wise

      600.37mg by area wise
- The percentage label claim for Cefuroxime axetil was 99.32% (height wise)and 100.89%(area wise) and for Linezolid 99.33% (areawise)
- The validation of the developed method was performed in accordance with ICH guidelines (Q2B Validation of Analytical Procedures: Methodology)
- The accuracy of the proposed method was studied by recovery studies at three levels (80%, 100 and 120%)

- The precision of the proposed method was studied by repeatability and intermediate precision. The %RSD of the proposed method was found to be < 2.
- The LOD and LOQ were determined and satisfactory results obtained.
- The proposed method was found to be accurate, precise and reliable.

6. CONCLUSION

A new method of analysis is developed for simultaneous estimation of Cefuroxime axetil and Linezolid drugs in pharmaceutical tablet dosage form by RP-HPLC and HPTLC method. The analytical procedure is validated as per ICH Q2B guidelines and shown to be simple,accurate, precise and specific. For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation step. HPLC and HPTLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool.

Cefuroxime axetil was freely soluble in ethanol, methanol, acetonitrile and insoluble in water. Linezolid was soluble in chloroform, alcohol and insoluble in ether. acetonitrile. and phosphate buffer was chosen as the mobile phase. The run time of the HPLC procedure was 10 minutes. The method was validated for system suitability, linearity, precision, accuracy, specificity, ruggedness robustness, LOD and LOQ. The system suitability parameters were within limit, hence it was concluded that the system was suitable to perform the assay. The method shows linearity between the concentration range of 10-50µg / ml. The % recovery of Cefuroxime axetil and Linezolid was found to be in the range of 99.22 % - 100.11 %. As there was no interference due to excipients and mobile phase, the method was found to be specific. The method was robust and rugged as observed from insignificant variation in the results of analysis by changes in flow rate and mobile phase composition

separately and analysis being performed by different analysts. Good agreement was seen in the assay results of Pharmaceutical formulation by developed method.

Hence, it can be concluded that the proposed methods using HPLC and HPTLC can be regarded as simple, fast reproducible and sensitive methods for the simultaneous estimation of Cefuroxime axetil and Linezolid in combined dosage form. Hence, these methods can be used for the in process evaluation in Pharmaceutical Manufacturing Firms and routine quality control of these drugs in Drug Testing Laboratories.

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