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# Analytical Method Development and Validation for Simultaneous Estimation of Avibactam and Ceftazidime by RP-HPLC Method

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**Abstract:**
**Background:**Literature review reveals that there is no analytical method reported for the analysis of Avibactam and ceftazidime by simultaneous estimation by RP-HPLC. Titrimetric analysis, HPLC and HPTLC are the reported analytical methods for compounds either individually or in combination with other dosage form.A simple reverse phase high performance liquid chromatography (RP-HPLC) method has been developed and subsequently validated for simultaneous determination of Avibactam andCeftazidime in combined dosage form. Ceftazidime and Avibactam is used to treat such gram – negative infection that are multidrug – resistant.
**Materials and methods:**The separation was carried out using mobile phase consisting of potassium dihydrogenortho phosphate buffer of pH 3.0 and Methanol in the ratio of 30:70 v/v. The column used is InertsilODS (5 µm, 4.6 X 150 mm) with flow rate of 1.0 ml/min using PDA detection at 260 nm. The calibration curves were linear over a concentration range of 1-5 µg/mL and 100-500 µg/mL for Avibactam and Ceftazidime. The retention times of were found to be 3.7 min and 2.05 min respectively. Results of analysis were validated statistically and by recovery studies. The LOD and LOQ value for Avibactam and Ceftazidime was found to be 3, 2.9ppm and 10.1, 10.03ppm respectively.
**Results:**The linear regression coefficient, slope and intercept of were found to be 0.997, 0999, 3003, 28847, 37313, 38353 respectively. The results of the study showed that the proposed RP-HPLC method is rapid, specific, precise and accurate and is useful for the routine analysis of Avibactam and Ceftazidime in bulk drug and in its pharmaceutical dosage form.
**Conclusion:**The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.
**Keywords:** Avibactam, Ceftazidime, RP-HPLC, Methanol

## I. INTRODUCTION

**ANALYTICAL CHEMISTRY:**
Analytical chemistry is a branch of chemistry specializing in the separation, identification and determination of the relative quantities of components comprising a sample of matter.
This is interested primarily in the qualitative analysis or compound identification and quantitative analysis of the compounds [1].

**CHROMATOGRAPHY**
Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. Chromatography may be preparative or analytical. The purpose of preparative Chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical Chromatography is done normally with smaller amounts of material and is for measuring the relative proportion of analyzes in a mixture [2] [5].

**HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)**
A variety of methods are available for analyzing pharmaceutical compounds. High Performance/Pressure Liquid Chromatography (HPLC) is one of the best methods of choice for analyzing a variety of natural and synthetic compounds. It is because it offers high performance over ambient pressure. [3]The phenomenal growth in chromatography is largely due to the introduction of the technique called high-pressure liquid chromatography, which is frequently called high-performance liquid chromatography (both are abbreviated as HPLC) [4,8].

**INSTRUMENTATION OF HPLC** [5, 7]

The individual components HPLC and their working functions are described below.

- Mobile phase and reservoir
- Solvent degassing system
- Pump
- Injector
- Column
- Detector
- Data system

**Figure 1: Schematic Diagram of HPLC**

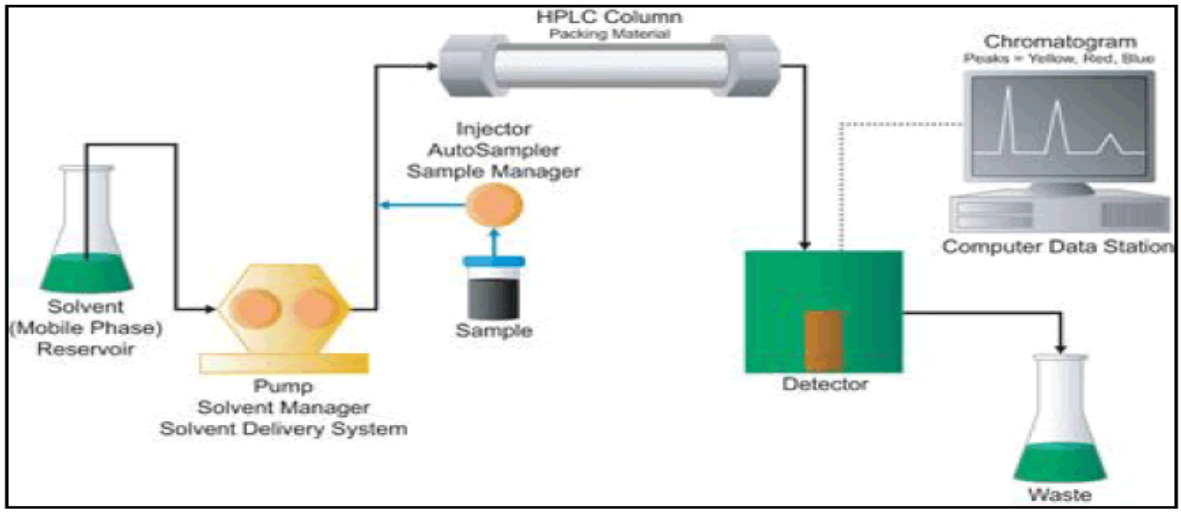



**Figure 1**. Block diagram of HPLC

**MOBILE PHASE AND RESERVOIR**

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing there is a high-pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity the polarity of the stationary phase and the nature of the sample components. For normal phase separations elute power increases with increasing polarity of the solvent but for reversed phase separations eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity [2].

Mobile phases used for HPLC typically are mixtures of organic solvents and water or aqueous buffers. Isocratic methods are preferable to gradient methods. Gradient methods will sometimes be required when the molecules being separated have vastly different partitioning properties. When a gradient elution method is used care must be taken to ensure that all solvents are miscible.

**SOLVENT RESERVOIR:**

The reservoir that holds the mobile phase is often no more than a glass bottle. Examples of mobile phase reservoirs range from standard laboratory glassware such as beakers or flasks covered with aluminum foil through larger vessels such as media bottles, solvent jugs, or carboys, to purpose-made glassware that includes built-in provision for stirring and degassing.

The requirements for a solvent reservoir are simple:

- ❖ Solvents should not be stored in plastic containers.
- ❖ Glass containers should not be used for aqueous mobile phase with pH>8.
- ❖ The reservoir and its attachment to the pump should be made of materials that will not contaminate the mobile phase: Teflon, glass, or stainless steel.
- ❖ In addition, a 10-micron frit or inlet filter called as “sinker frit” should be connected to the end of the inlet line that dips into the reservoir.

**SOLVENT DEGASSING SYSTEM:**

Mobile phase accounts for 70 % or more of all the problems in chromatography. Presence of particles in mobile phase may cause non producible flow rate, decrease in selectivity, spurious peaks, increased back pressure, irreversible adsorption and decrease in life of column. Hence, 0-20-micron size filters are being used for filtration. Degassing is one of the most effective measures to eliminate these problems. Benefits are:

- Stability in baseline, enhanced sensitivity
- Reproducible retention times for eluting peaks
- Reproducible injection volumes for quantization.
- Stable pump operation

The methods for Degassing include:

**1. Sonication:** When pure solvents are mixed to make up the mobile phase, excess dissolved gas escapes to form bubbles. If the mobile phase reservoir is placed in an ultrasonic bath, the sound waves promote the coalescence of small bubbles which can escape more easily. Sonication alone will degas a gallon of solvent approximately in 20 minutes.

**2. Vacuum or offline degassing**: vacuum reduces pressure of gases on the surface of the solvent which reduces the mass of gas in the solution. Advanced system called the Inline vacuum degassers are developed where the mobile phase is passed through gas permeable tubing enclosed in a vacuum chamber such that the air free mobile phase directly enters the pump without any resaturation of air.

**3. Helium sparge:** A stream of helium bubbles will sweep dissolved air out of liquids (helium is virtually insoluble in most HPLC solvents, so very little helium replaces the air). Helium sparing is very effective & is suitable for use with on-line mixing systems. This removes background absorbance on UV detectors and the quenching phenomenon caused by the dissolved oxygen on a fluorescent detector.

**4. Heat or offline degassing method:** This method increases the vapour pressure of the solution which proportionately decreases the partial pressure of the gas in the solution. This will prevent the further absorption of the gas into the solution.

Heat may reduce the solubility of the gas in solution. But this method is not suitable for organic solvents.

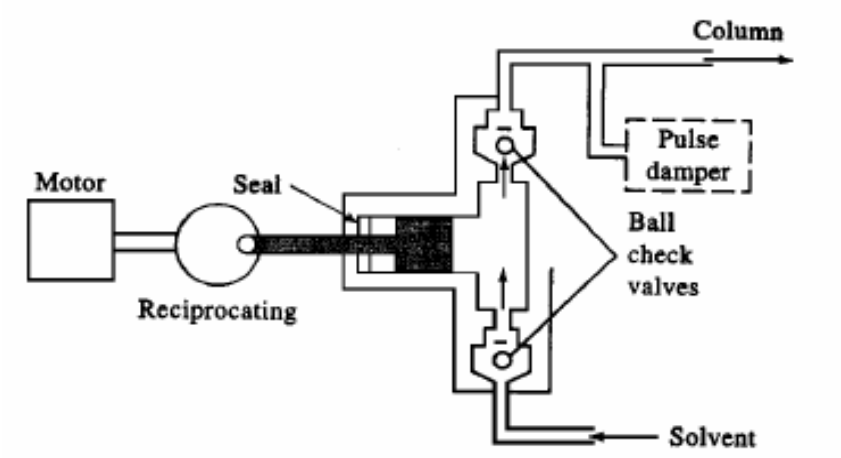
**5. On-line membrane degassing**: The mobile phase or solvent flows through a hollow fiber made of a semi-permeable membrane. A partial vacuum is maintained on the outside of the membrane. Because air can diffuse through the membrane while solvent vapor cannot, dissolved air is removed from the solvent before it reaches the pump.

**PUMP:**

The pump is one of the most important components of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system.

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

**Figure 2: Schematic Diagram of Reciprocating Pump**



**INJECTOR:**

Insertion of the sample onto the pressurized column in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. It is always best to remove particles from the sample by filtering over a 5 µm filter or centrifuging since continuous injections of particulate material will eventually cause blockages in injection devices or columns. Sample sizes may vary widely.

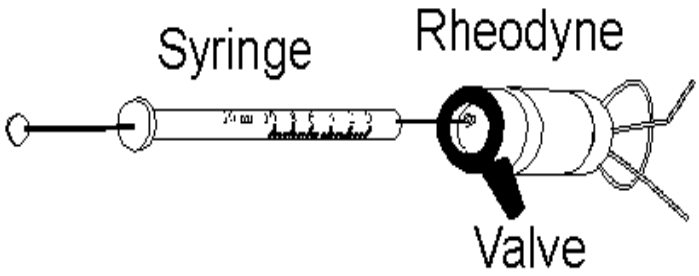
There are three important ways of introducing the sample into injection port:

➔ **Loop injection:**In which, a fixed amount of volume is introduced by making use of fixed volume loop injector.

➔ **Valve injection:**In which, a variable volume is introduced by making use of an injection valve.

➔ **On column injection:**In which, a variable volume is introduced by means of a syringe through a septum.

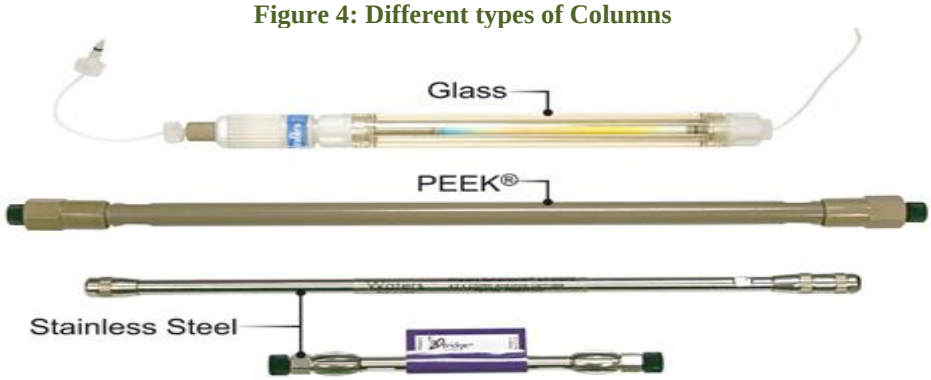
**Figure 3: Rheodyne Injector**




**COLUMN:**

The heart of the system is the column. The packing used in modern HPLC consists of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

**Figure 4: Different types of Columns**




**1. Porous, polymeric beds:** Porous, polymeric beds based on styrene divinely benzene co-polymers used in ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

**2. Porous layer beds:** Consisting of a thin shell (1-3 µm) of silica or modified silica on a spherical inert core (e.g. Glass). After the development of totally porous micro particulate packing, these have not been used in HPLC.

**3. Totally porous silica particles (dia. < 10 µm):** These packing have widely been used for analytical HPLC in recent years. Particles of diameter >20 µm are usually dry packed, while particles of diameter <20 µm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.

**Table 1:commonly used columns for method development and validation**

| S.NO | MFG COMPANEY | COLUMN BRAND NAME | PARTICAL SIZE (µm) | COLUMN DIMENSIONS (mm) | TYPE OF COLUMN |
|---|---|---|---|---|---|
| 1. | Waters | X-Terra | 5 µm | 4.6(i.d) ×250mm | C18 |
| 2. | ACE | Ace HPLC | 5 µm | 4.0(i.d) ×250mm | C18 |

| | | | | | |
|---|---|---|---|---|---|
| | | Column | | | |
| 3. | Lichrochart | Lichrospher | 5 µm | 4.0(i.d) ×250mm | C18 |
| 4. | GL Sciences | Inertsil ODS | 5 µm | 4.6(i.d) ×250mm | C18 |
| 5. | Lmtakt | Unison US | 5 µm | 4.6(i.d) ×250mm | C18 |
| 6. | Waters | Symmetry Shield | 5 µm | 4.6(i.d) ×250mm | C18 |
| 7. | Waters | Spherisorb | 5 µm | 4.6(i.d) ×250mm | C18 |
| 8. | Zodiac Life Sciences | Zodiac | 5 µm | 4.6(i.d) ×250mm | C18 |
| 9. | Nomura Chemical | Develosil ODS | 5 µm | 4.6(i.d) ×250mm | C18 |
| 10. | Grace | Kromasil | 5 µm | 4.6(i.d) ×150mm | C18 |
| 11. | Thermo | Hypersil BDS | 5 µm | 4.6(i.d) ×150mm | C18 |
| 12. | Waters | X-Terra | 5 µm | 4.6(i.d) ×150mm | C8 |

**DETECTOR:**

The detector converts a change in the column effluent into an electrical signal that is recorded by the data system. There are different types of detectors used in HPLC. Liquid chromatographic detectors are of two basic types [10]. Bulk property detectors respond to a mobile-phase bulk property, such as refractive index, dielectric constant, or density. In contrast, Solute property detectors respond to some property of solutes, such as UV absorbance, fluorescence, or diffusion current, that is not possessed by the mobile phase.

**A**) **Refractive index detector**:The detection principle involves measuring of the change in refractive index of the column effluent passing through the flow-cell. The greater the RI difference between sample and mobile phase, the larger the imbalance will become. Thus, the sensitivity will be higher for the higher difference in RI between sample and mobile phase. On the other hand, in complex mixtures, sample components may cover a wide range of refractive index values and some may closely match that of the mobile phase, becoming invisible to the detector.

**B) UV Detector**: In these systems detection depends on absorption of UV ray energy by the sample. They are capable to detect very wide range of compounds. The sensitivity ranges till microgram quantity of estimation.

**C) PDA detector**: These are detectors which follow principle similar to UV detectors but the only advantages are higher sensitivity and measure the entire absorption range i.e. It gives scan of entire spectrum.

**D) Evaporative Light Scattering Detector (ELSD):** In the ELSD, the mobile phase enters the detector is evaporated in a heated device and the remaining solute is finally detected by the way it scatters light. The intensity of the light scattered from solid suspended particles depends on their particle size. Therefore, the response is dependent on the solute particle size produced. This, in turn, depends on the size of droplets generated by the nebulizer and the concentration of solute in the droplets. The droplet size produced in the instrument nebulizer depends on the physical properties of the liquid and the relative velocity and flow-rates of the gas and liquid stream. The importance of all these parameters emphasizes the need for careful design and rigorous optimization of the instrument parts.

**E) Electro Chemical Detector:** This detector is especially suitable to estimate oxidizable& reducible compounds. The principle is that when compound is either oxidized or reduced, the chemical reaction produces electron flow. This flow is measured as current which is the function of type and quantity of compound

**F) Conductivity Detector:**Conductivity detector measures the conductivity of the mobile phase. There is usually background conductivity which must be backed-off by suitable electronic adjustments. If the mobile phase contains buffers, the detector gives a base signal that completely overwhelms that from any solute usually making detection impossible. Thus, the electrical conductivity detector is a bulk property detector and senses all ions whether they are from a solute or from the mobile phase.

**G)**Fluorescence detectors: Fluorescence detectors are probably the most sensitive among the existing modern HPLC detectors. It is possible to detect even a presence of a single analytic molecule in the flow cell. Typically, fluorescence sensitivity is 10 -1000 times higher than that of the UV detector for strong UV absorbing materials. Fluorescence detectors are very specific and selective among the others optical detectors. This is normally used as an advantage in the measurement of specific fluorescent species in samples.

**H)** Mass Spectrometric Detection: The use of mass spectrometer for HPLC detection is becoming common place, despite the high cost of such detector and need for a skilled operator. A mass spectrometer can facilitate HPLC method development and avoid common problem by

- Tracking and identifying individual peaks in the chromatogram between experiments
- Distinguishing compounds of interest from minor compounds or interferences.

- Recognizing unexpected and overlapping interference peaks to avoid a premature finish to method development.

**DATA SYSTEM:**

Since the detector signal is electronic using modern data collection techniques can aid the signal analysis. The main goal in using electronic data systems is to increase analysis accuracy and precision while reducing operator attention.

**Applications of HPLC** [2][4][5][6]

- Preparative HPLCrefers to the process of isolation and purification of compounds.
- Chemical separationscan be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates for a given set of column and mobile phase.
- Purification refers to the process of separating or extracting the target compound from other (possibly structure related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic condition. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.
- Identification of the compoundsby HPLC is a crucial part of any HPLC assay. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from the extraneous peaks at the detection levels in which the assay would be performed.
- Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a solution. It involves injecting a series of known concentration of the standard compound solution onto the HPLC for detection. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected

**METHOD VALIDATION**

Analytical Method Validation can be defined as (ICH) "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

Method validation study include system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments [9].

**SPECIFICITY**

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

**LINEARITY**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity studies should cover the range of 0-150% of the expected level of the analyte. The data is then processed using the method of least squares regression. The resulting plot, slope, intercept and correlation coefficient provide the desired information on linearity. ICH recommends that, for the establishment of linearity, a minimum of five concentrations should normally be used.

**ACCURACY**

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. There are three ways to determine accuracy:

- Comparison to a reference standard
- Recovery of the analyte spiked into blank matrix
- Standard addition of the analyte

Accuracy is calculated as the percentage of 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, together with the confidence intervals.

ICH documents recommend that accuracy should be assessed using a minimum of nine determinations i.e., three replicates at three concentrations across the specified range of the procedure.

**PRECISION**

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

**a. Repeatability**

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

**b. Intermediate precision**

Intermediate precision expresses within laboratories variations: different day's different analysts, different equipment, etc.

**c. Reproducibility**

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

ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range of the procedure (i.e., three replicates of three concentrations) or using a minimum of six determinations at 100% of the test concentration.

**DETECTION LIMIT (LOD)**

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

- Based on Visual Evaluation.
- Based on Signal-to-Noise.
- Based on Standard Deviation of the Response and the Slope.

LOD can be expressed as:

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

Where, σ = Standard deviation of intercepts of calibration curves

S= Mean of slopes of the calibration curves

The slope S may be calculated from the calibration curve of the analyte.

**QUANTITATION LIMIT (LOQ)**

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Several approaches for determining the Quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on Visual Evaluation
- Based on Signal-to-Noise
- Based on Standard Deviation of the Response and the Slope

LOQ can be expressed as:

$$\mathbf{LOQ = 10\sigma/S}$$

Where,

σ = Standard deviation of intercepts of calibration curves.

S= Mean of slopes of the calibration curves.

The slope S may be calculated from the calibration curve of the analyte.

**RANGE**

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range of the analytical procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy and linearity when applied to the samples containing analytes at the extremes of the range as well as within the range.

**ROBUSTNESS**

The robustness of an analytical procedure 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. A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters may involve temperature ($\pm 5^0$C), buffer $p^H$ (±0.5), ionic strength of buffers, level of additives to MP, flow rate (±0.2ml/min), wavelength (±2nm).

**RUGGEDNESS:**
The precision obtained when the assay is performed by multiple analysis, using multiple instruments, on multiple days, in one laboratory, different sources of reagents and multiple lots of columns should also be included in this study.

## II. LITERATURE REVIEW

**Rabindra K et al**., A reverse phase high performance liquid chromatography (HPLC) method has been developed for the simultaneous determination of Ceftazidime Sodium and Tazobactam Sodium in pharmaceutical dosage form. HPLC was carried out on a BDS Hypersil C18 column (5 µm, 250 X 4.6 mm ID) using Acetonitrile: 0.02 M potassium dihydrogen phosphate buffer pH 3.5 with orthophosphoric acid (10% aqueous) with 2 drops of TEA in the ratio of 80:20 (v/v) as the mobile phase at a flow rate of 1.0 mL/min and eluents were monitored at 254 nm. The calibration curves were linear over the range of 50 – 200 µg/mL for Ceftazidime Sodium and 5-30 µg/mL for Tazobactam Sodium. The retention time of Ceftazidime Sodium and Tazobactam Sodium was found to be 3.0 min and 5.4 min respectively. The accuracy and precision of the methods were determined and validated statistically. All the methods showed good reproducibility and found to be rapid, specific, precise and accurate with % RSD less than 2. These methods can be successfully applied for the routine analysis of CEFTA and TAZO in bulk and combined dosage form. Keywords: Reverse Phase High Performance Liquid Chromatography; Ceftazidime Sodium; Tazobactam

**Andréia de Haro Moreno et al**., three different assays for the quality control of ceftazidime in commercial formulations were developed and compared: acidimetric, iodometric and nonaqueous methods. Method validation yielding good results and included precision and accuracy, with good recovery percent ranged from 99.67 to 100.39, and R.S.D. values smaller than 2%. Results were compared to those obtained by high performance liquid chromatographic method, developed and published previously by us, and no evidence of significant difference was observed. The results obtained agreed well with the contents stated on the labels, being rapid, simple and inexpensive alternative method for the determination of ceftazidime in pharmaceutical formulations.

**R.K. Nanda et al**., This paper describes a method developed and validated using high performance thin layer chromatography (HPTLC) for the simultaneous estimation of Ceftazidime Sodium (CEFTA) and Tazobactam Sodium (TAZO) in a combined dosage form. Procedure does not require prior separation of components from the sample. The method was carried out in TLC Precoated silica gel on aluminum plate 60 F 254 (0.2 mm thickness, 10 cm ×10 cm, prewashed by methanol and activated at 60° C for 5 min prior to chromatography). The solvent system was Chloroform: Ethyl acetate: Glacial acetic acid: Water in the proportion of 4:4:4:1.8, (v/v/v/v) with RF value for CEFTA and TAZO was 0.16 and 0.45 respectively. Calibration curves were established showing the dependence of response (peak area) on the amount chromatographed. The validated linearity ranges were 500–2500 ng /spot (r2 = 0.999) and 10–62.5 µg/spot (r2 = 0.998) for CEFTA and TAZO respectively. The spots were scanned at λ =254 nm. The suitability of this HPTLC method for quantitative determination of the compounds was proved by validation in accordance with the requirements of the ICH guidelines. The method was used for determination of the compounds in commercial pharmaceutical dosage forms. The method is simple, reproducible, accurate and can be used as a more economical alternative to other chromatographic techniques for routine quality control.

**Dave Vimal M et al**., A reverse phase high performance liquid chromatographic method was developed for the simultaneous estimation of Cefepime and Amikacin in injection formulation. The separation was achieved by C18 (250 x 25mm) 25µm column and Acetonitrile: water (10:90 v/v) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at 212 nm. Retention time of Amikacin and Cefepime was found to be 2.51 min and 6.23 min, respectively. The method has been validated for linearity, accuracy and precision. Linearity for Cefepime and Amikacin were in the range of 20-100 µg/ml. The percentage recoveries obtained for Cefepime and Amikacin were found to be in range of 98.22±0.56 and 99.63±0.57 respectively. Developed method was found to be accurate, precise, selective and rapid for simultaneous estimation of Cefepime and Amikacin in injection. The proposed method was successfully applied for the simultaneous estimation of both drugs in commercial injection preparation.

**Rabindra K. Nanda et al.,** A reverse phase high performance liquid chromatography (HPLC) method has been developed for the simultaneous determination of Ceftazidime Sodium and Tazobactam Sodium in pharmaceutical dosage form. HPLC was carried out on a BDS Hypersil C18 column (5 µm, 250 X 4.6 mm ID) using Acetonitrile: 0.02 M potassium dihydrogen phosphate buffer pH 3.5 with orthophosphoric acid (10% aqueous) with 2 drops of TEA in the ratio of 80:20 (v/v) as the mobile phase at a flow rate of 1.0 mL/min and eluents were monitored at 254 nm. The calibration curves were linear over the range of 50 – 200 µg/mL for Ceftazidime Sodium and 5-30 µg/mL for Tazobactam Sodium. The retention time of Ceftazidime Sodium and Tazobactam Sodium was found to be 3.0 min and 5.4 min respectively. The accuracy and precision of the methods were determined and validated statistically. All the methods showed good reproducibility and found to

be rapid, specific, precise and accurate with % RSD less than 2. These methods can be successfully applied for the routine analysis of CEFTA and TAZO in bulk and combined dosage form.

## III. DRUG PROFILE

**AVIBACTAM**

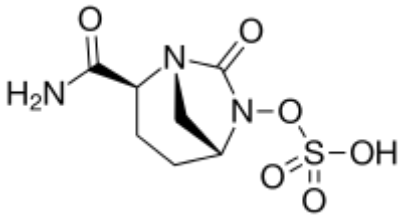


**Figure 5: Chemical Structure of Avibactam**

**IUPAC Name:** [(2S, 5R)-2-Carbamonyl-7-oxo-1, 6-diazabicyclo [3.2.1] octan-6-yl] hydrogen sulfate.
**Molecular Formula:** $C_7H_{11}N_3O_6S$
**Molecular weight:** 265.24 g/mol
**State:**A crystalline solid
**Solubility:** soluble in DMSO
**Category:** Non-β-lactam β-lactamase inhibitor

**Mechanism of action** Emerging β-lactamase-mediated resistance is threatening the clinical utility of the single most prominent class of antibacterial agents used in medicine, the β-lactams. The diazabicyclooctane avibactam is able to inhibit a wider range of serine β-lactamases than has been previously observed with β-lactamase inhibitors such as the widely prescribed clavulanic acid. However, despite its broad-spectrum activity, variable levels of inhibition have been observed for molecular class D β-lactamases. In order to better understand the molecular basis and spectrum of inhibition by avibactam, we provide structural and mechanistic analysis of the compound in complex with important class A and D serine β-lactamases. Herein, we reveal the 1.7- and 2.0-Å-resolution crystal structures of avibactam covalently bound to class D β-lactamases OXA-10 and OXA-48. Furthermore, a kinetic analysis of key active-site mutants for class A β-lactamase CTX-M-15 allows us to propose a validated mechanism for avibactam-mediated β-lactamase inhibition including a unique role for S130, which acts as a general base. This study provides molecular insights that will aid in the design and development of avibactam-based chemotherapeutic agents effective against emerging drug-resistant microorganisms.

**CEFTAZIDIME**

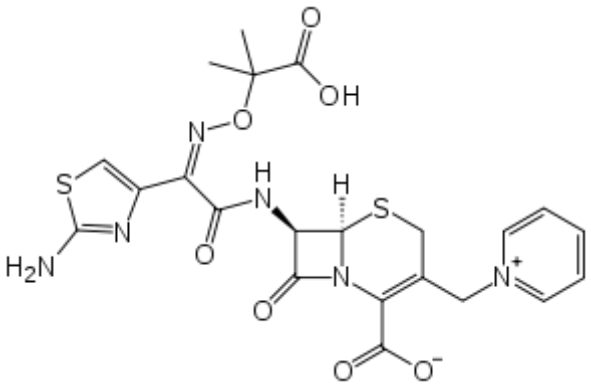


**Figure 6: Chemical Structure of Ceftazidime**

**IUPAC Name:**(6*R*, 7R, *Z*)-7-(2-(2-aminothiazol-4-yl)-2-(2-carboxypropan-2-yloxyimino) acetamido)-8-oxo-3-(pyridinium-1-ylmethyl)-5-thia-1-aza bicycle [4.2.0] oct-2-ene-2-carboxylate**.**
**Molecular Formula:** $C_{22}H_{22}N_6O_7S_2$
**Molecular weight:** 546.58g/mol
**Description:**Semi synthetic, broad-spectrum antibacterial derived from cephaloridine and used especially for Pseudomonas and other gram-negative infections in debilitated patients.
**State**: Solid

**Solubility:** soluble in water
**Category**: Cephalosporin Antibiotic

**Mechanism of action:** The bactericidal activity of ceftazidime results from the inhibition of cell wall synthesis via affinity for penicillin-binding proteins (PBPs).

## IV. EXPERIMENTAL METHODOLOGY

**INSTRUMENTS USED**

**Table 2: List of Instruments used**

| SL. No | Instrument | Model |
|---|---|---|
| 1 | HPLC | WATERS, software: Empower, 2695 separation module, PDA detector. |
| 2 | UV/VIS spectrophotometer | LABINDIA UV $3000^{+}$ |
| 3 | pH meter | Adwa – AD 1020 |
| 4 | Weighing machine | Afcoset ER-200A |

**CHEMICALS USED:**

**Table 3: List of Chemicals used**

| SL. No | Chemical | Brand |
|---|---|---|
| 1 | ceftazidime | Boehringer |
| 2 | Avibactam | Cipla |
| 3 | $KH_2PO_4$ | FINER chemical LTD |
| 4 | Water and Methanol for HPLC | LICHROSOLV (MERCK) |
| 5 | Acetonitrile for HPLC | MOLYCHEM |
| 6 | Ortho phosphoric Acid | MERCK |

**HPLC METHOD DEVELOPMENT:**

**Mobile Phase Optimization**:

Initially the mobile phase tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to potassium dihydrogen phosphate with buffer (pH 3.0), Methanol in proportion 30: 70 %v/v respectively.

**Wave length selection**:

UV spectrum of 10 µg / ml Avibactam and ceftazidime in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 260nm. At this wavelength both the drugs show good absorbance.

**Optimization of Column:**

The method was performed with various columns like C18 column, hypersil column, Xterra, Symmetry and inertsil ODS column. Inertsil ODS (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1 .0ml/min flow.

**OPTIMIZED CHROMATOGRAPHIC CONDITIONS**:

Instrument used : Waters HPLC with auto sampler and PAD or detector.
Temperature : Ambient
Column : Inertsil ODS (4.6 x 150mm, 5µm)
Buffer : 6.8 grams of potassium dihydrogen ortho phosphate in 1000 ml water pH adjusted with ortho phosphoric acid.
pH : 3.0
Mobile phase : 30% buffer 70% Methanol
Flow rate : 1.0 ml per min
Wavelength : 260 nm
Injection volume : 10 µl
Run time : 10min.

**PREPARATION OF BUFFER AND MOBILE PHASE:**

**Preparation of Phosphate buffer:**

Accurately weighed 6.8 grams of $KH_2PO_4$ was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with Orthophosphoric acid.

**Preparation of mobile phase:**

Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

**Diluent Preparation:**

The Mobile phase was used as the diluent.

**PREPARATION OF THE CEFTAZIDIME& AVIBACTAM STANDARD & SAMPLE SOLUTION:**

**Standard Solution Preparation:**

Accurately weigh and transfer 10 mg of Avibactam and ceftazidime10mg of working standard into a 10mL& 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 3ml& 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

**Sample Solution Preparation:**

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10 mg ofAvibactam and ceftazidime(marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 3 ml of Ceftazidime e and Avibactam of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Procedure:**

Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Avibactam and ceftazidimepeaks and calculate the %Assay by using the formula.

**SYSTEM SUITABILITY:**

Tailing factor for the peaks due to Avibactam and ceftazidimein Standard solution should not be more than 2.0

Theoretical plates for the Avibactam and ceftazidimepeaks in Standard solution should not be less than 2000.

**Calculation:**

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

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 mg/ml.

**METHOD VALIDATION:**

**PRECISION:**

**Preparation of stock solution:**

Accurately weigh and transfer 10 mg of Avibactam and ceftazidime working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 3 ml of Ceftazidime& Avibactam of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Procedure:**
The standard solution was injected for five times and measured the area for all five Injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.
**Acceptance Criteria:**
The % RSD for the area of five standard injections results should not be more than 2%**.**

**INTERMEDIATE PRECISION/RUGGEDNESS:**
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

**Preparation of stock solution:**
Accurately weigh and transfer 10 mg of Ceftazidime and 10mg of Avibactam working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution)
Further pipette 3ml of Ceftazidime&Avibactam **of** the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.
**Procedure:**
The standard solution was injected for five times and measured the area for all five
injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.
**Acceptance Criteria:**
The % RSD for the area of five standard injections results should not be more than 2%.

**ACCURACY:**
**Preparation of Standard stock solution:**
Accurately weigh and transfer 10 mg of Avibactam and ceftazidime10mg of working standard into a 10mL& 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution)
Further pipette 3ml& 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

**Preparation Sample solutions:**
**For preparation of 50% solution (With respect to target Assay concentration):**
Accurately weigh and transfer 5mg of Ceftazidime and 5.3mg of Avibactam working standard into a 10mL and 100 ml 0f clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock Solution).
Further pipette 3 ml of Ceftazidime e & 0.3 ml of Avibactam of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**For preparation of 100% solution (With respect to target Assay concentration):**
Accurately weigh and transfer 10 mg of Ceftazidime and 10 mg of Avibactam working standard into a 10mL and 100 ml 0f clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock Solution).
Further pipette 3 ml of Ceftazidime & 0.3 ml of Avibactam of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**For preparation of 150% solution (With respect to target Assay concentration):**
Accurately weigh and transfer 14.4mg of Ceftazidime and 14.5mg of Avibactam working standards into a 10mL and 100ml of clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).
Further pipette 3 ml of Ceftazidime& 0.3 ml of Avibactam of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Procedure:**
Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150%
solutions. Calculate the Amount found and Amount added for Ceftazidime &Avibactam **and** calculate the individual recovery and mean recovery values.

**Acceptance Criteria:**

- The % Recovery for each level should be between 98.0 to 102.0%.

**LINEARITY:**

**Preparation of stock solution:**

Accurately weigh 10 tablets crush in mortar and pestle and transfer equivalent to 10 mg ofAvibactam and ceftazidime(marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

**Preparation of Level – I (100ppm ofCeftazidime&1ppm of Avibactam):**

1ml and 0.1 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

**Preparation of Level – II (200ppm of Ceftazidime&2ppm of Avibactam):**

2ml and 0.2 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

**Preparation of Level – III (300ppm of Ceftazidime&3ppm of Avibactam):**

3ml and 0.3 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

**Preparation of Level – IV (400ppm of Ceftazidime&4ppm of Avibactam):**

4ml and 0.4 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

**Preparation of Level – V (500ppm of Ceftazidime&5ppm of Avibactam)**

5ml and 0.5 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

**Procedure:**

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

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

**Acceptance Criteria:**

- Correlation coefficient should be not less than 0.999.

**LIMIT OF DETECTION:**

**Limit of Detection: (For Ceftazidime):**

**Preparation of 300µg/ml solution:**

Accurately weigh and transfer 10 mg of Ceftazidime working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

(Stock solution)

Further pipette 3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Preparation of 0.12µg/ml solution):**

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

Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent

Pipette 0.4mL of 1µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

**Calculation of S/N Ratio:**

Average Baseline Noise obtained from Blank : 52µV

Signal Obtained from LOD solution : 152 µV

S/N = 152/52 = 2.9

**Acceptance Criteria:**

- S/N Ratio value Shall be 3 for LOD solution.

**Limit of Detection: (For Avibactam)**
**Preparation of 3µg/ml solution:**
Accurately weigh and transfer 10mg of Avibactam working standard into a 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution)
Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Preparation of 0.015µg/ml solution):**
Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.
Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Calculation of S/N Ratio:**
Average Baseline Noise obtained from Blank : 52 µV
Signal Obtained from LOD solution : 156 µV
S/N = 156/52 = 3.0

**Acceptance Criteria:**
- S/N Ratio value shall be 3 for LOD solution.

**LIMIT OF QUANTIFICATION:**
**Limit of Quantification (for Ceftazidime)**
**Preparation of 300µg/ml solution:**
Accurately weigh and transfer 10 mg of Ceftazidime working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Preparation of 0.42µg/ml solution):**
Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.
Pipette 1.0mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.
Pipette 1.4 mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

**Calculation of S/N Ratio:**
Average Baseline Noise obtained from Blank : 52 µV
Signal Obtained from LOQ solution : 522µV

S/N = 522/52 = 10.03

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

**Limit of Quantification:(for Avibactam)**
**Preparation of 3µg/ml solution:**
Accurately weigh and transfer 10mg of Avibactam working standard into a 100mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution)
Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

**Preparation of 0.05µg/ml solution):**
Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.
Pipette 1.7mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

**Calculation of S/N Ratio:**
Average Baseline Noise obtained from Blank : 52 µV
Signal Obtained from LOQ solution : 524µV
S/N = 524/52 = 10.

**ROBUSTNESS:**
As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

**The flow rate was varied at 0.8 ml/min to 1.2ml/min.**
Standard solution 300ppm of Ceftazidime& 3ppm of Avibactam was prepared and analysed using the varied flow rates along with method flow rate.
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%.

***** Results for actual flow (1.0ml/min) have been considered from Assay standard.

**The Organic composition in the Mobile phase was varied from 50% to 50%.**
Standard solution 300 µg/ml of Ceftazidime& 3µg/ml of Avibactamwas prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.On evaluation of the above results, it can be concluded that the variation in 10%.Organic composition in the mobile phase affected the method significantly. Hence it Indicates that the method is robust even by change in the Mobile phase ±10
***** Results for actual Mobile phase composition (70:30%v/v Methanol: Buffer (pH-3.0) has been considered from Assay stand.

## V. RESULTS AND DISCUSSION

**OPTIMIZED CHROMATOGRAM IS OBTAINED BY FOLLOWING CONDITIONS:**
**TRIAL 1:**

| | | |
|---|---|---|
| Mobile phase | : | Water: Methanol (50:50%v/v) |
| Column | : | Xterra C18 (4.6*250mm) 5µm |
| Flow rate | : | 1.0 ml/min |
| Wavelength | : | 260 nm |
| Column temp | : | Ambient |
| Sample Temp | : | Ambient |
| Injection Volume | : | 10 µl |

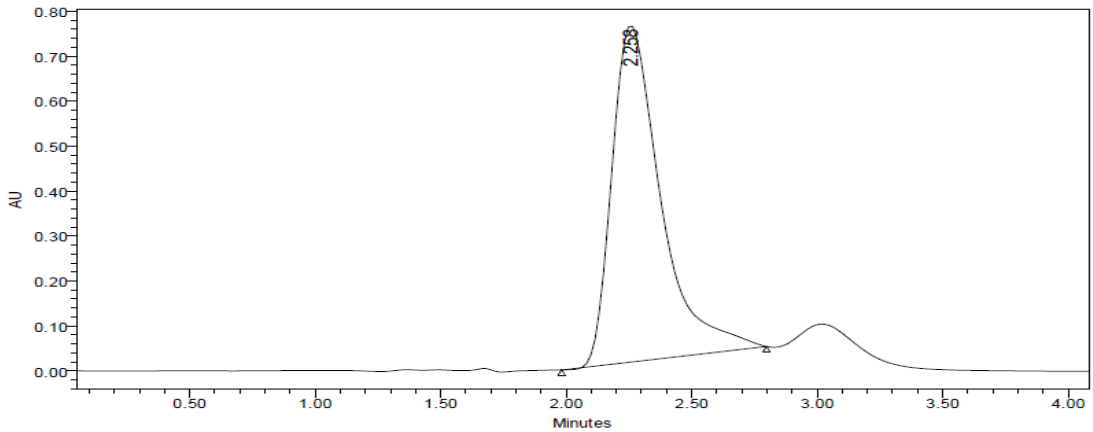



**Figure 6: Chromatogram for Ceftazidime and Avibactam – Trial-1**

**Observation:**
From the above chromatogram it was observed that the ceftazidime peak was splitted

**TRIAL 2:**
Mobile phase : Phosphate buffer (0.05m) pH 4.0: Methanol (40:60%v/v)
Column : Xterra C18 (4.6*250mm) 5µm
Flow rate : 1.0 ml/min
Wavelength : 260 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume : 10 µl

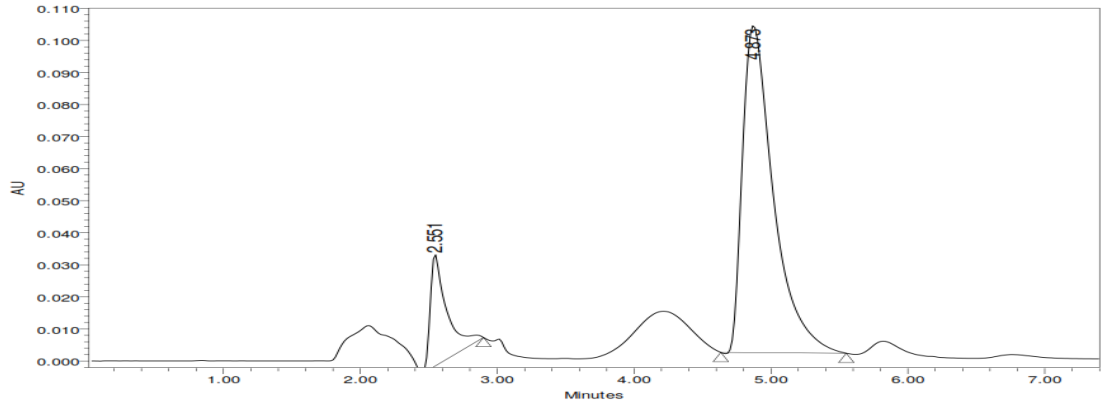



**Figure 7: Chromatogram for Ceftazidime and Avibactam – Trial-2**

**Observation:**
From the above chromatogram it was observed that the ceftazidime and Avibactam peaks are spitted and tailing was observed.

**TRIAL 3:**
Mobile phase : Phosphate buffer (0.05m) pH 4.0: Methanol (40:60%v/v)
Column : Symmetry C18 5µm (4.6*250mm) Make; waters
Flow rate : 0.8 ml/min
Wavelength : 260 nm
Column temp : Ambient
Sample Temp : Ambient
Injection Volume : 10 µl

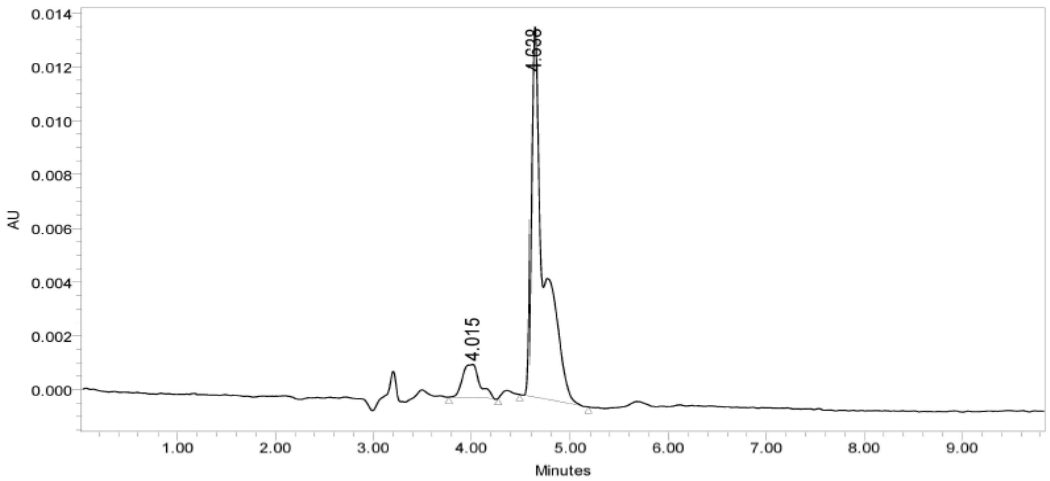



**Figure 8:Chromatogram for Ceftazidime and Avibactam – Trial-3**

**Observation:**
From the above chromatogram it was observed that the ceftazidime and Avibactam peaks are splitted

**TRIAL 4:**

| | | |
|---|---|---|
| Mobile phase | : | Phosphate buffer (0.05M) pH 3.6: ACN (40:60%v/v) |
| Column | : | Symmetry C18 5µm (4.6*250mm) Make; waters |
| Flow rate | : | 0.8 ml/min |
| Wavelength | : | 260 nm |
| Column temp | : | Ambient |
| Sample Temp | : | Ambient |
| Injection Volume | : | 10 µl |

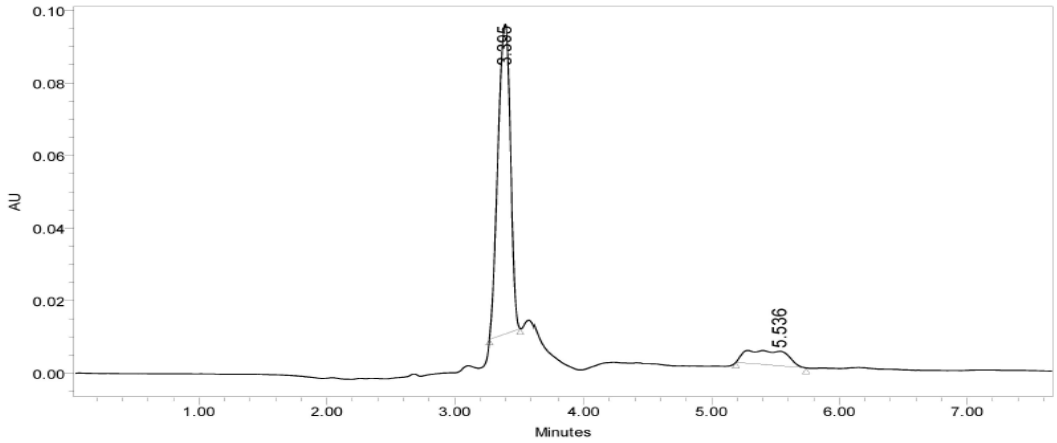



**Figure 9:Chromatogram for Ceftazidime and Avibactam – Trial - 4**

**Observation:**
From the above chromatogram it was observed that the ceftazidime and Avibactam peaks are splitted with tailing.

**TRIAL 5:**

| | | |
|---|---|---|
| Mobile phase | : | Phosphate buffer pH 3.0: Methanol (30:70%v/v) |
| Column | : | Inertial C18 5µm (4.6*250mm) |
| Flow rate | : | 0.8 ml/min |
| Wavelength | : | 260 nm |
| Column temp | : | Ambient |
| Sample Temp | : | Ambient |
| Injection Volume: | | 10 µl |

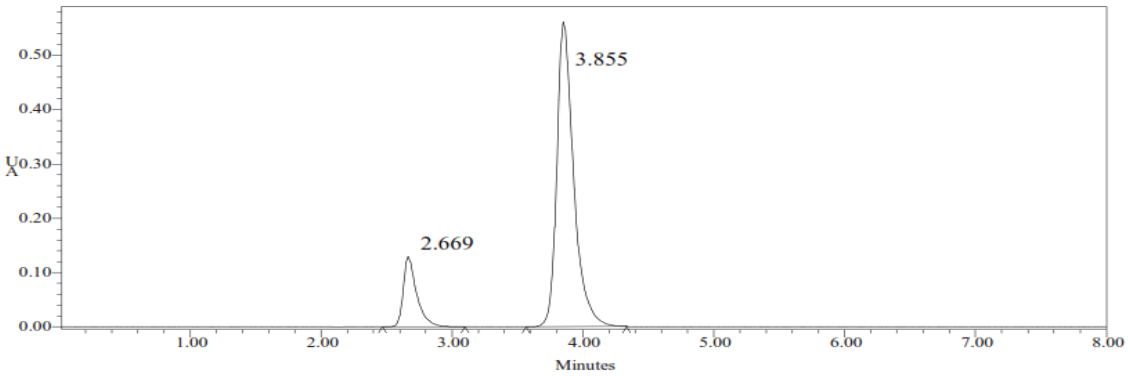



**Figure 10: Chromatogram for Ceftazidime and Avibactam – Trial-5**

**Observation:**
From the above chromatogram it was observed that the ceftazidime and Avibactam peaks are splitted

**Chromatogram for Ceftazidime and Avibactam (Optimized)**

| | | |
|---|---|---|
| Column | : | Inertial C18 (4.6 x 250mm, 5µm) |
| Buffer pH | : | 3.0. |
| Mobile phase | : | 30% buffer 70% Methanol |
| Flow rate | : | 1.0ml per min |
| Wavelength | : | 260 nm |
| Temperature | : | ambient. |
| Run time | : | 10min. |

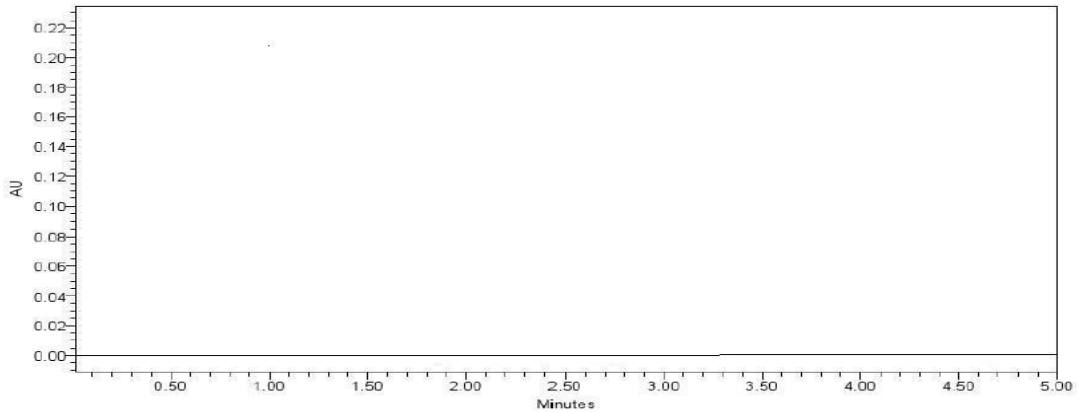



**Figure 11: Chromatogram for blank**

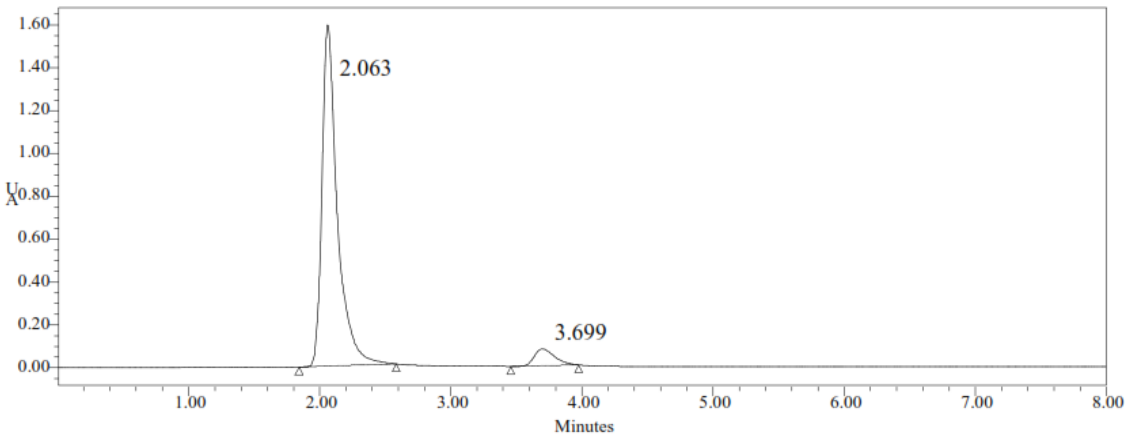



**Figure 12:Chromatogram for Ceftazidime and Avibactam (Sample)**

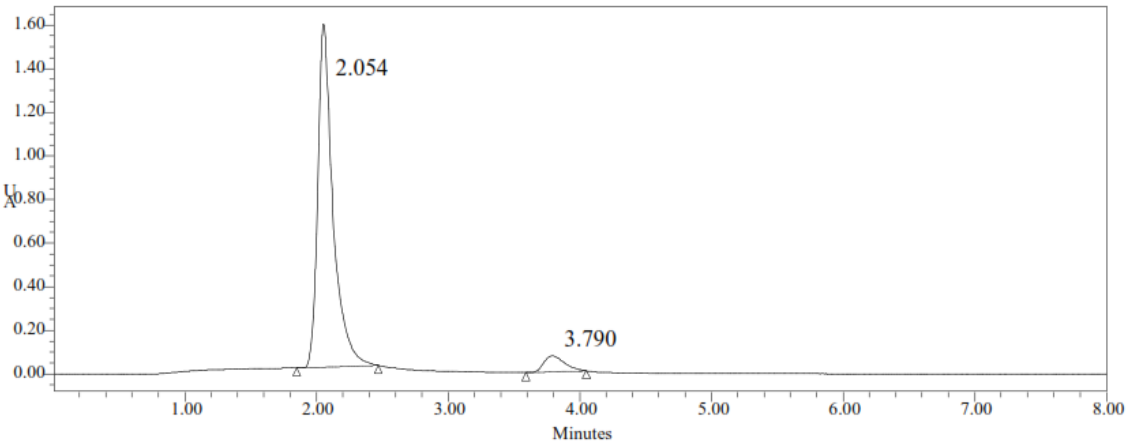



**Figure 13:Chromatogram for Ceftazidime and Avibactam (Standard)**

**SYSTEM SUITABILITY:**

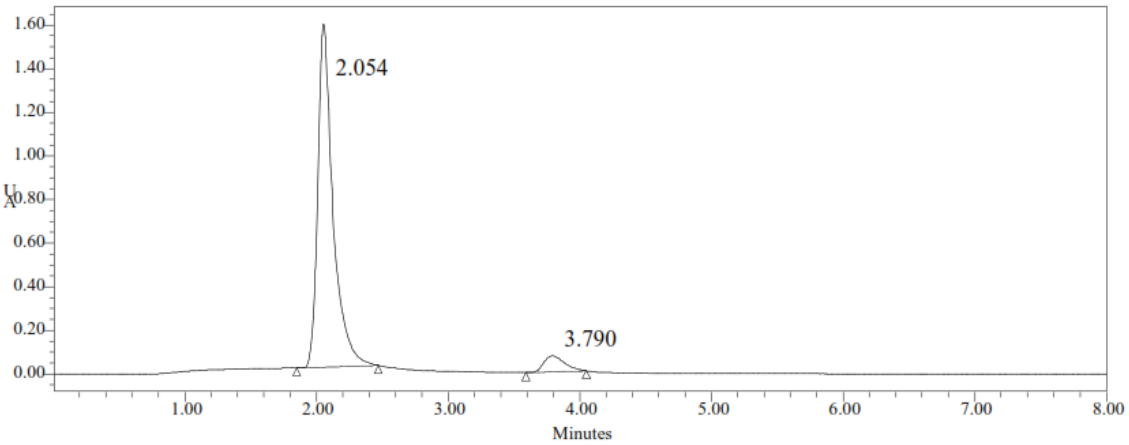



**Figure 14: Chromatogram for system suitability**

**Calculation: (ForCeftazidime)**

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

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 ceftazidime mg/ml.

**RESULTS:**

**System Suitability Results:**

1). Tailing factor Obtained from the standard injection is 1.3
2). Theoretical Plates Obtained from the standard injection is 4668.7

**Assay Results:**

Weight of 10 tablets: 1.25 grams
Average Weight : 0.125grams

$$= \frac{1314011}{1311967} \times \frac{10}{10} \times \frac{3}{10} \times \frac{10}{25} \times \frac{10}{3} \times \frac{99.8}{100} \times \frac{125}{50} \times 100 = 99.95\%$$

**Calculation: (For Avibactam)**

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

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 Avibactam mg/ml.

**RESULTS:**

**System Suitability Results:**

1). Tailing factor Obtained from the standard injection is 1.3

2). Theoretical Plates Obtained from the standard injection is 6090.3

**Assay Results:**

Weight of 10 tablets: 1.25 grams

Average Weight: 0.125grams

$$= \frac{125260}{124581} \times \frac{10}{100} \times \frac{0.3}{10} \times \frac{10}{25} \times \frac{10}{3} \times \frac{99.7}{100} \times \frac{125}{0.5} \times 100 = 100.24\%$$

**Results of system suitability parameters for ceftazidime and Avibactam**

**Table 4: system suitability parameters for ceftazidime and Avibactam**

| S.No | Name | Retention time(min) | Area (µV sec) | Height (µV) | USP resolution | USP tailing | USP plate count |
|---|---|---|---|---|---|---|---|
| 1 | **Ceftazidime** | 2.05 | 124505 | 213642 | | 1.2 | 4673.4 |
| 2 | **Avibactam** | 3.7 | 1308495 | 154566 | 6 0 | 1.3 | 6090.3 |

**Acceptance criteria:**

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

**VALIDATION PARAMETERS:**

**PRECISION:**

Precision of the method was carried out for standard solutions as described under experimental work. The corresponding chromatograms and results are shown below.

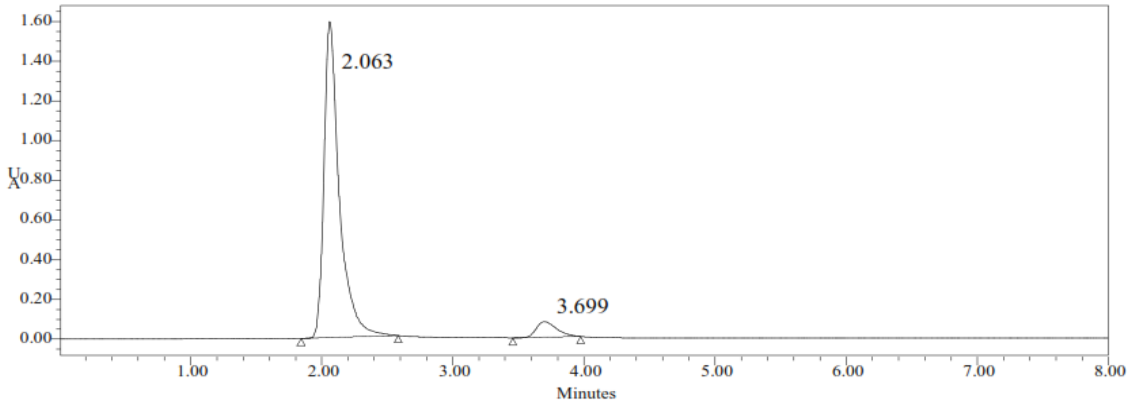



**Figure 15: Chromatogram for method Precision study Injection -1**

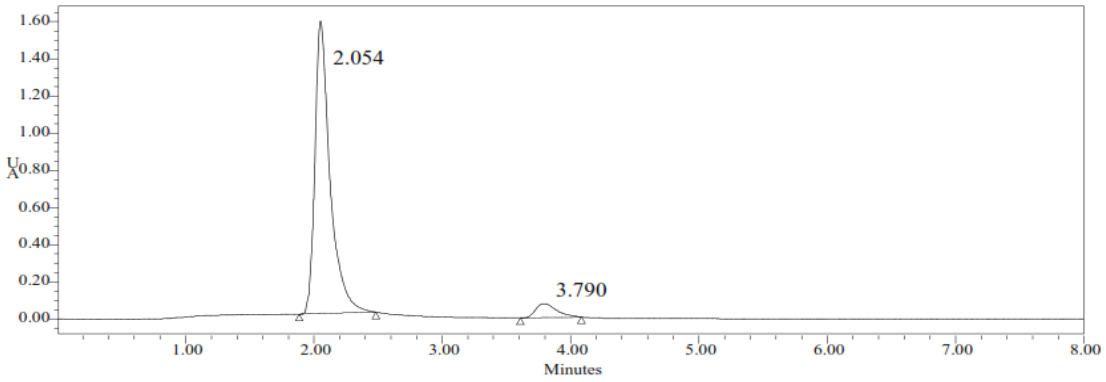



**Figure 16: Chromatogram for method Precision study Injection -2**

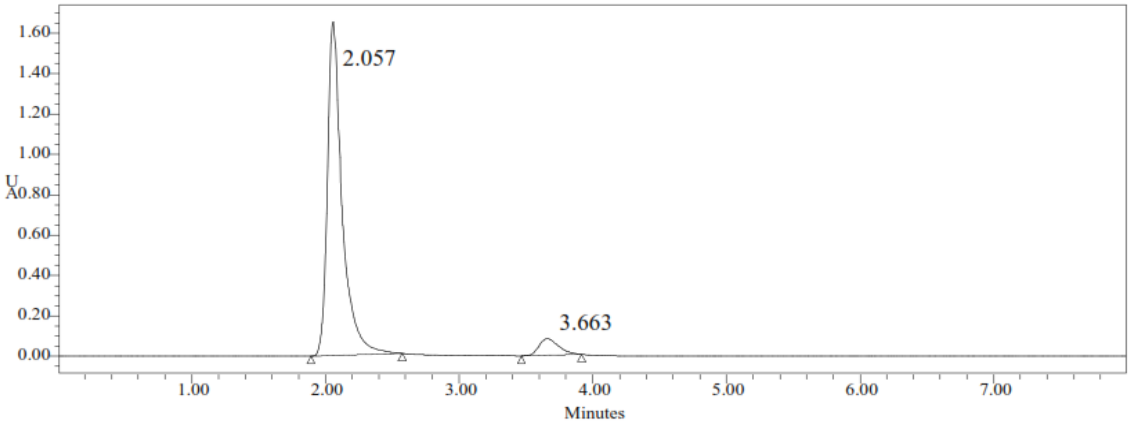



**Figure 17: Chromatogram for method Precision study Injection -3**

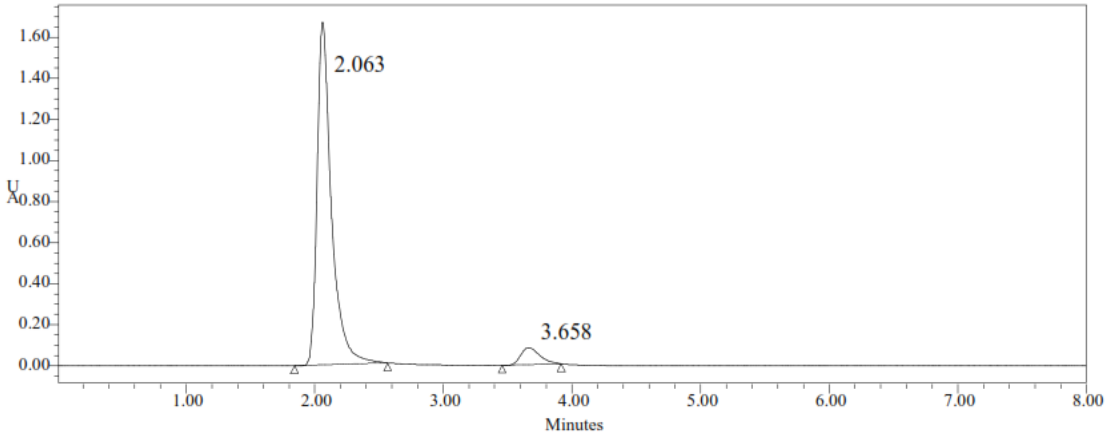



**Figure 18: Chromatogram for method Precision study Injection -4**

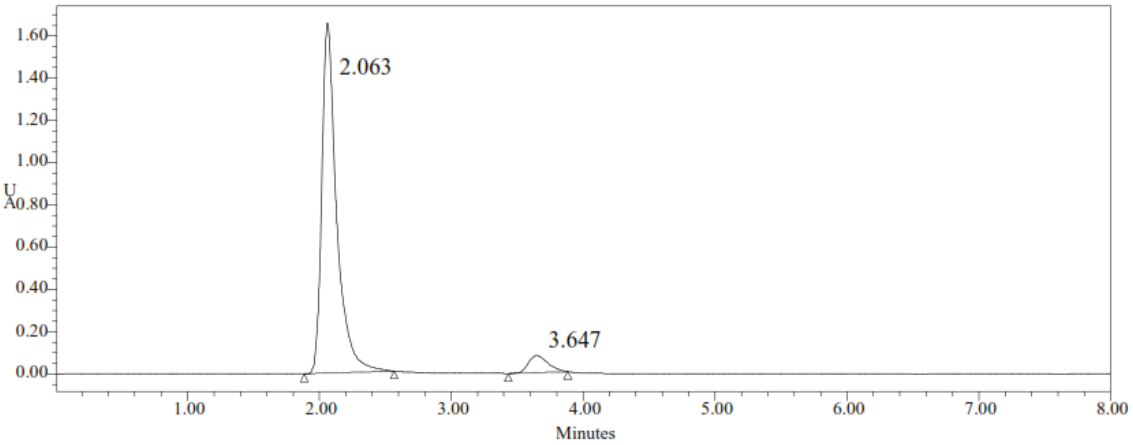



**Figure 19: Chromatogram for method Precision study Injection -5**

**Table 5: Results of method precession for ceftazidime**

| **Injection** | **Area** |
|---|---|
| Injection-1 | 1302729 |
| Injection-2 | 1302947 |
| Injection-3 | 1303236 |
| Injection-4 | 1303977 |
| Injection-5 | 1309759 |
| Average | 1304529.8 |
| Standard Deviation | 2961.1 |
| %RSD | 0.2 |

**Table 6: Results of method precession for Avibactam**

| **Injection** | **Area** |
|---|---|
| Injection-1 | 123149 |
| Injection-2 | 123766 |
| Injection-3 | 124271 |
| Injection-4 | 124691 |
| Injection-5 | 124956 |
| Average | 124162.7 |
| Standard Deviation | 725.6 |
| %RSD | 0.6 |

**Acceptance criteria:**

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

**INTERMEDIATE PRECESSION (RUGGEDNESS):**

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation.

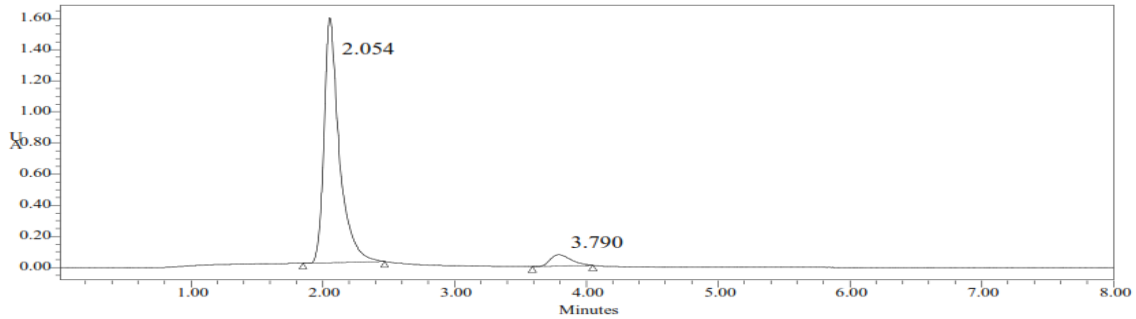



**Figure 20: Chromatogram for Precision study Injection -1**

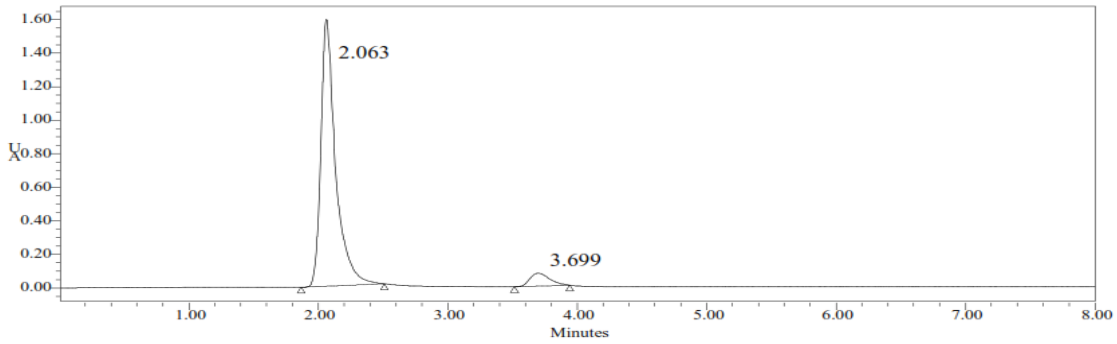



**Figure 21: Chromatogram for Precision study Injection -2**

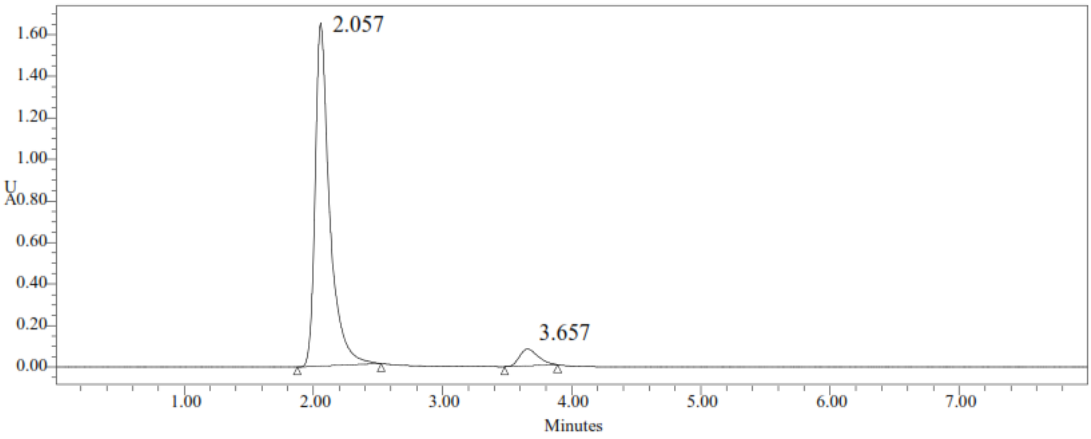



**Figure 22: Chromatogram for Precision study Injection -3**

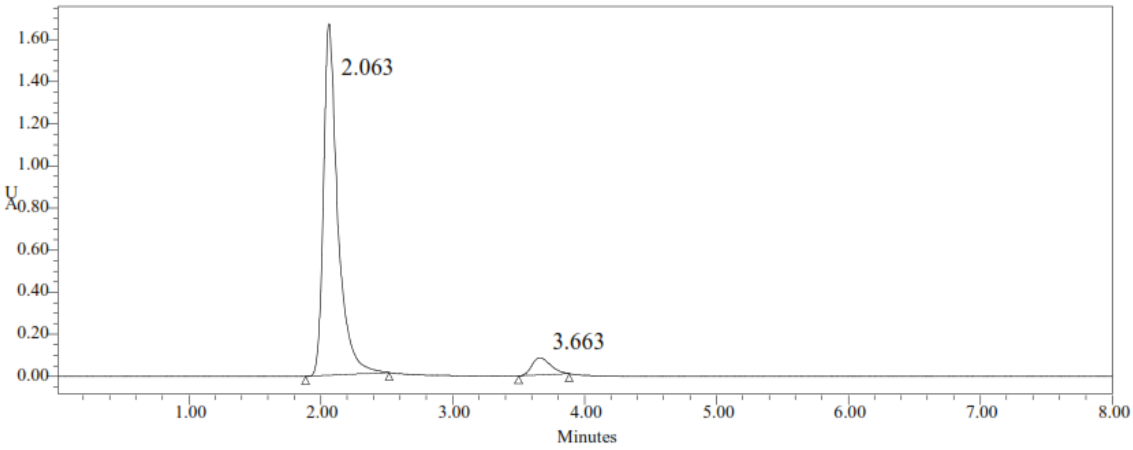



**Figure 23: Chromatogram for Precision study Injection -4**

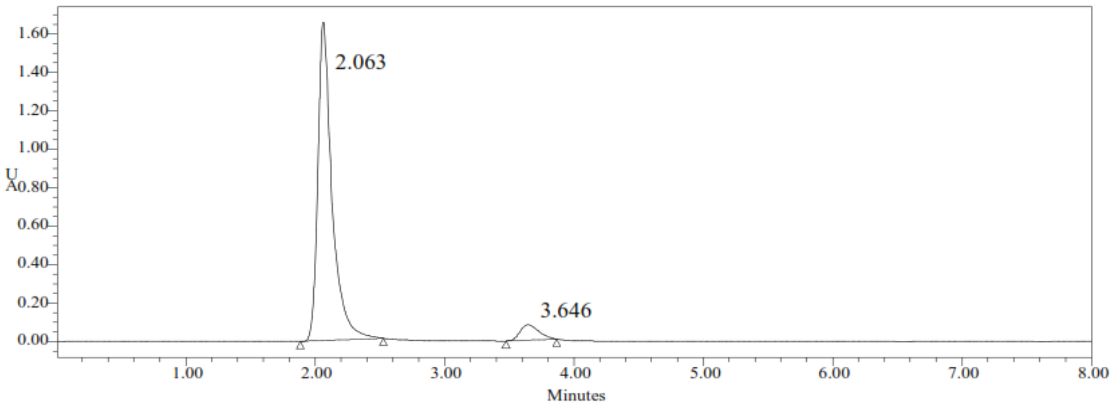



**Figure 24: Chromatogram for Precision study Injection -5**

**TABLE 7: Results of Intermediate precision for Ceftazidime**

| **Injection** | **Area** |
|---|---|
| Injection-1 | 1300148 |
| Injection-2 | 1304520 |
| Injection-3 | 1305937 |
| Injection-4 | 1306476 |
| Injection-5 | 130871 |
| Average | 1305070.2 |
| Standard Deviation | 3061.8 |
| %RSD | 0.2 |

**TABLE 8: Results of Intermediate precision for Avibactam**

| **Injection** | **Area** |
|---|---|
| Injection-1 | 122487 |
| Injection-2 | 122626 |
| Injection-3 | 122632 |
| Injection-4 | 122702 |
| Injection-5 | 122962 |
| Average | 122681.8 |
| Standard Deviation | 174.8 |
| %RSD | 0.1 |

**Acceptance criteria:**

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

**ACCURACY:**

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

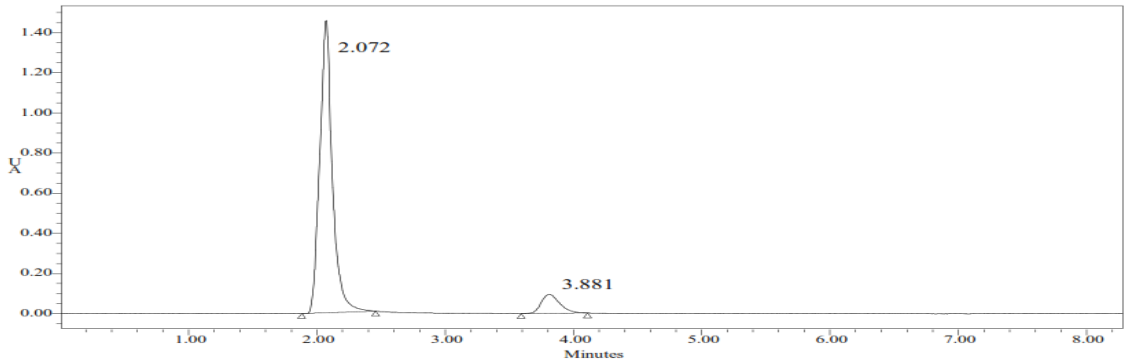



**Figure 25 (a):Chromatogram for accuracy level concentration-50% - I**

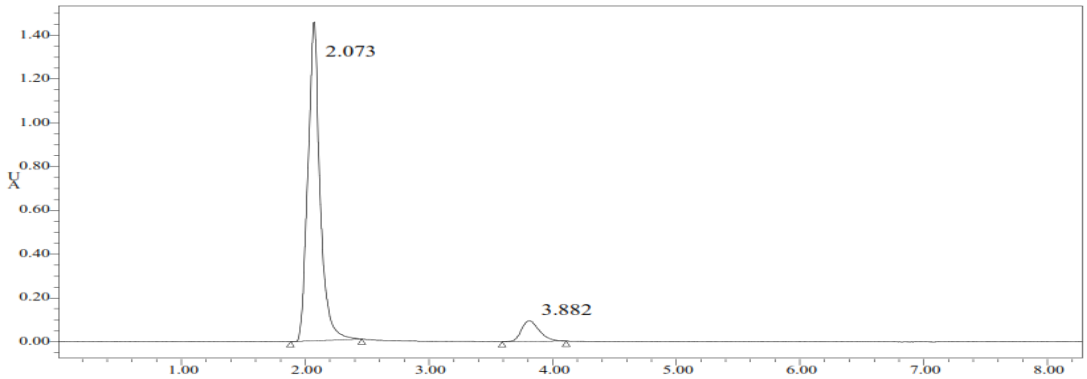



**Figure 25 (b): Chromatogram for accuracy level concentration-50% - II**

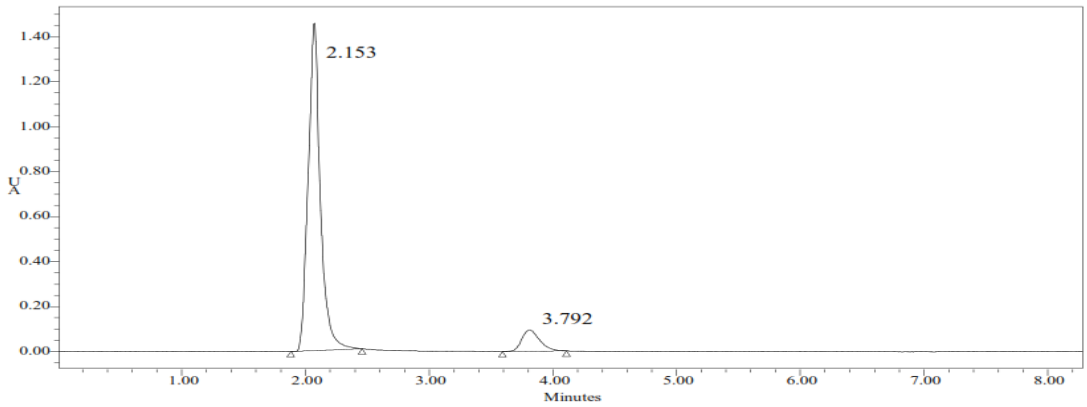



**Figure 25 (c): Chromatogram for accuracy level concentration-50% - III**

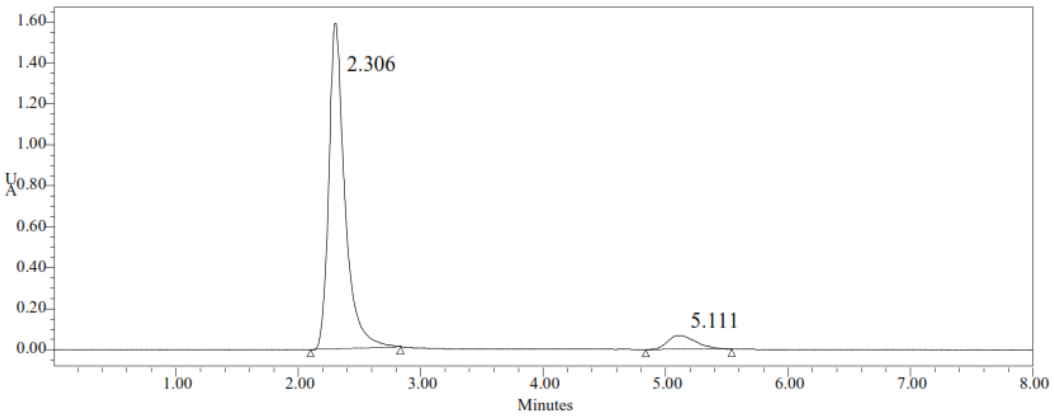



**Figure 26 (a): Chromatogram for accuracy level concentration-100% - I**

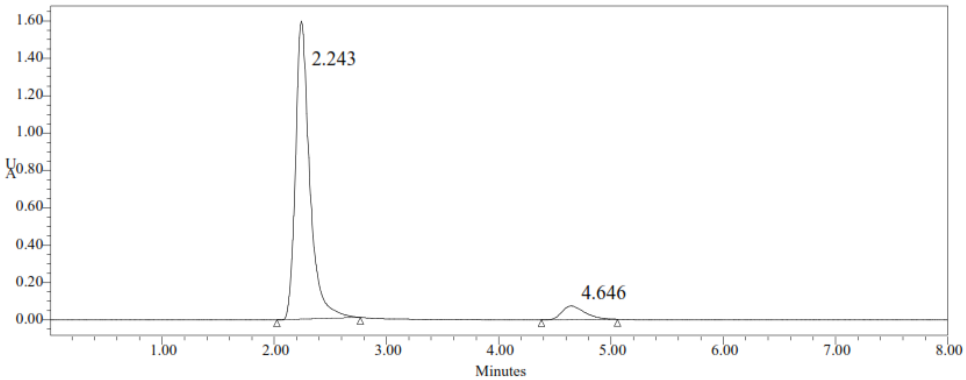



**Figure 26(b): Chromatogram for accuracy level concentration-100% - II**

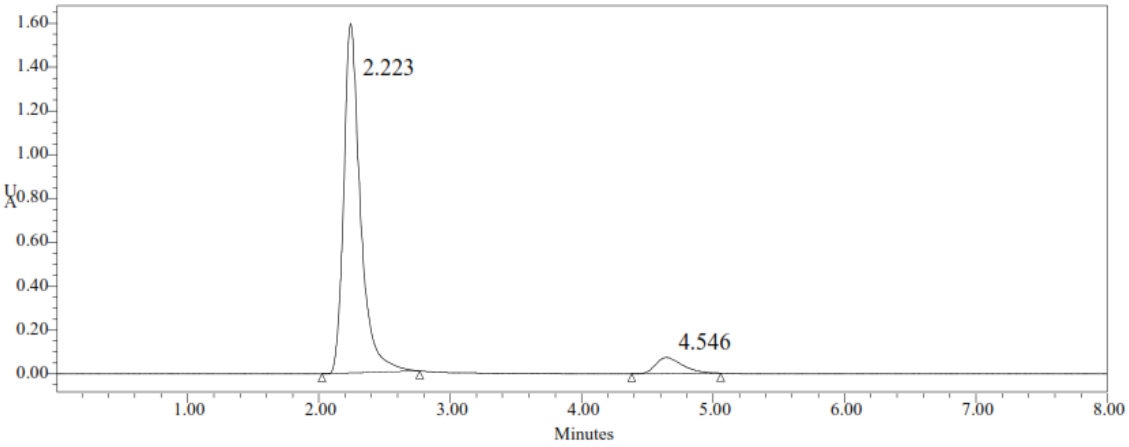



**Figure 26(c): Chromatogram for accuracy level concentration-100% - III**

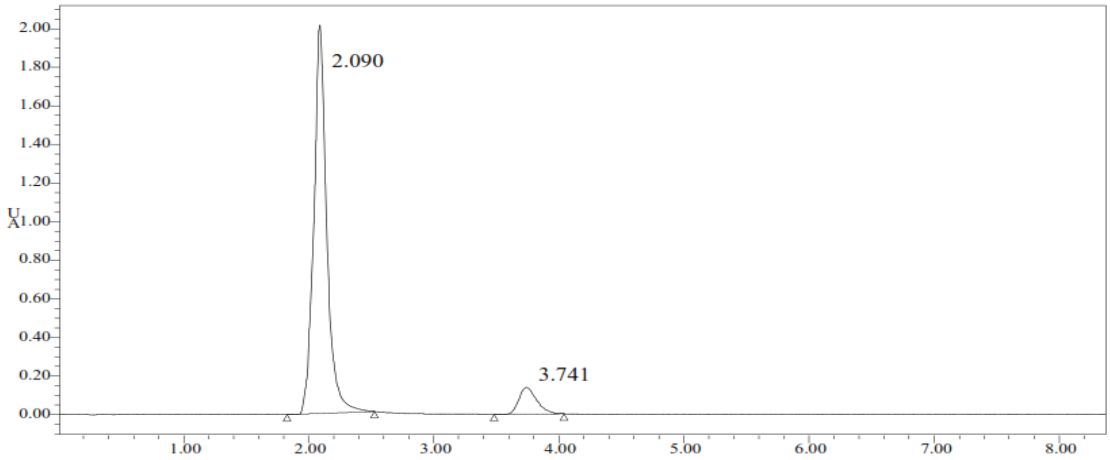



**Figure 27(a): Chromatogram for accuracy level concentration -150%-I**

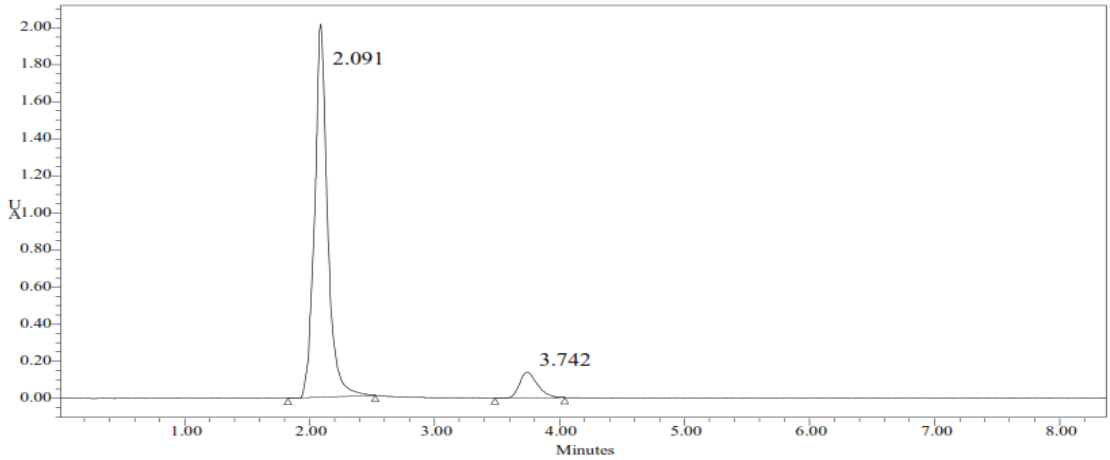



**Figure 27(b): Chromatogram for accuracy level concentration -150%-II**

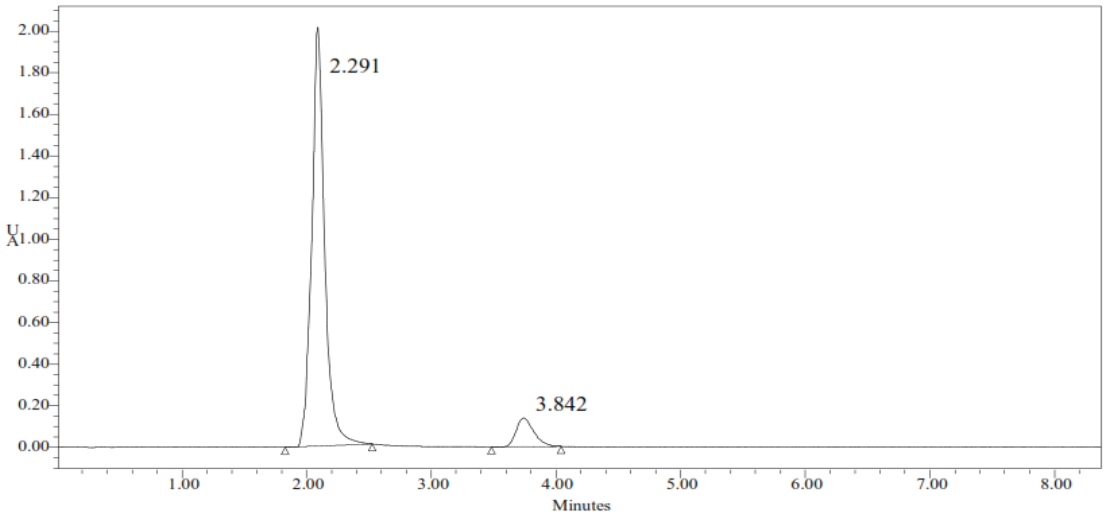



**Figure 27(c): Chromatogram for accuracy level concentration -150%-III**

**Table 9: Accuracy (recovery) results for Ceftazidime**

| %Concentration (at specification Level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery |
|---|---|---|---|---|---|
| 50% | 656659.5 | 5.0 | 5.036 | 100.7% | 99.84% |
| 100% | 1304258 | 10.0 | 10.003 | 100.0% | |
| 150% | 1854608 | 14.4 | 14.224 | 98.780% | |

**Table 10: Accuracy (recovery) results for Avibactam**

| %Concentration (at specification Level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery |
|---|---|---|---|---|---|
| 50% | 65800 | 5.3 | 5.34 | 100.8% | 100.51% |
| 100% | 124353 | 10 | 10.10 | 100.01% | |
| 150% | 177940 | 14.2 | 14.45 | 99.68% | |

**Acceptance Criteria:**

- The % Recovery for each level should be between 98.0 to 102.0%.
- The percentage recovery was found to be within the limit (97-103%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate

**LINEARITY:**

The linearity range was found to lie from 100µg/ml to 500µg/ml of ceftazidime, 1µg/ml to 5µg/ml of Avibactam and chromatograms are shown below.

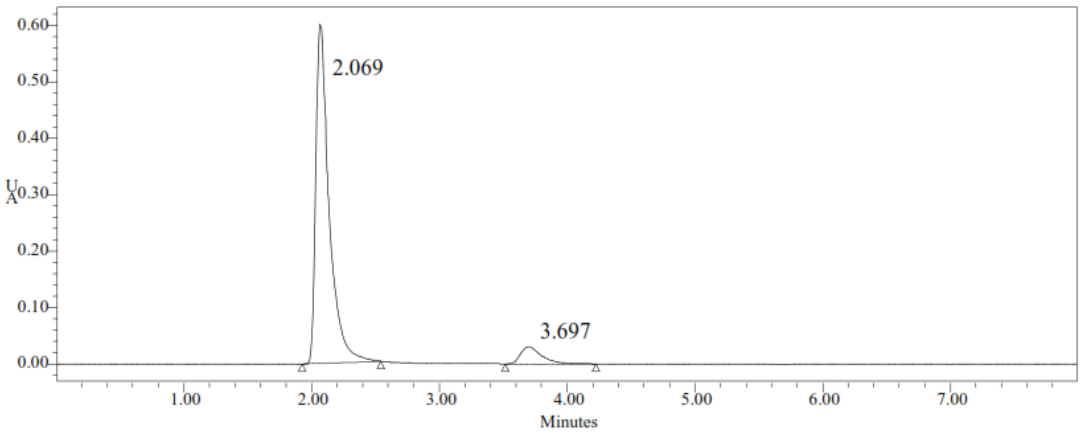



**Figure 28: Chromatogram of Ceftazidime and Avibactam - linearity level -I**

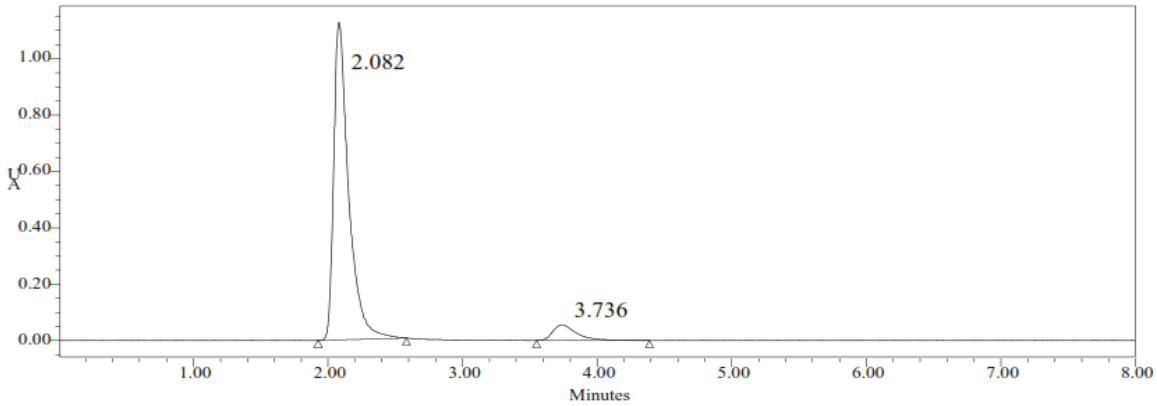



**Figure 29: Chromatogram of Ceftazidime and Avibactam - linearity level –II**

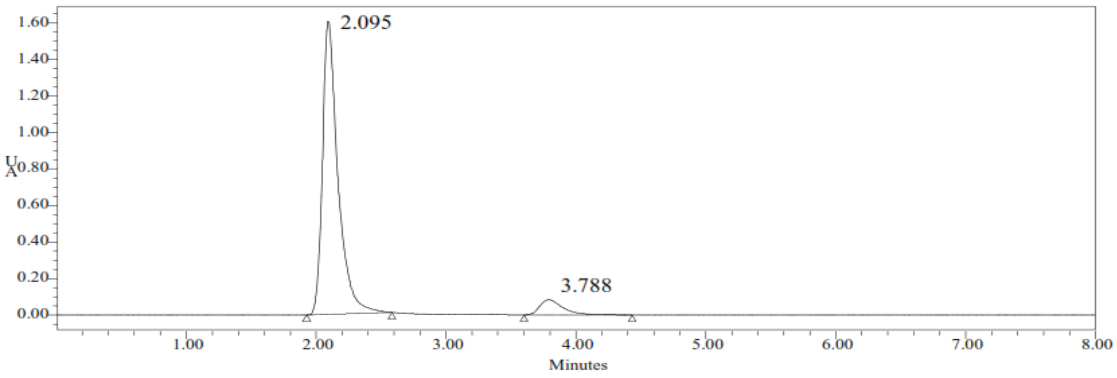



**Figure 30: Chromatogram of Ceftazidime and Avibactam - linearity level -III**

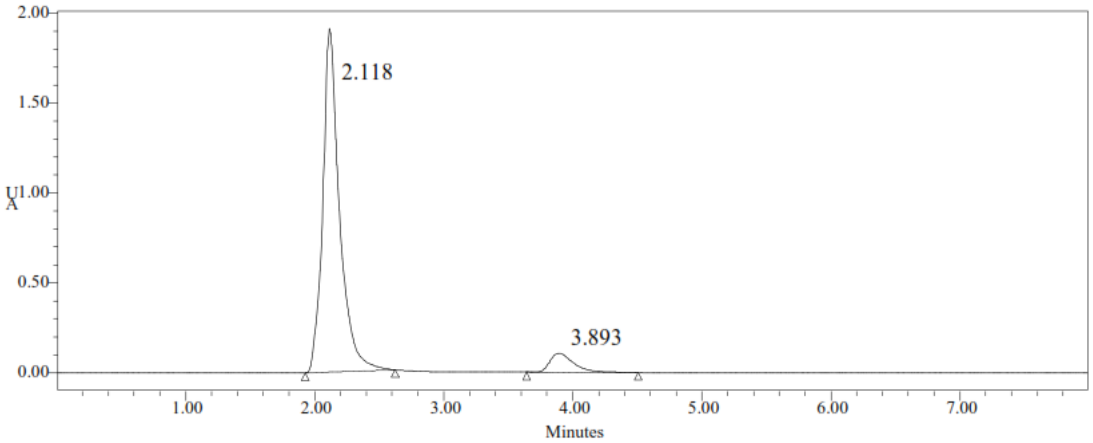



**Figure 31: Chromatogram of Ceftazidime and Avibactam - linearity level -IV**

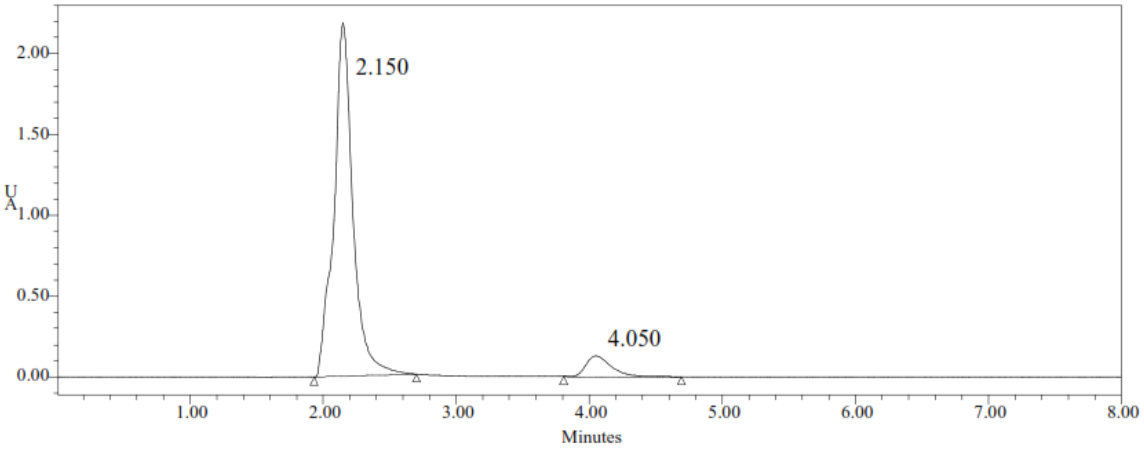



**Figure 32: Chromatogram of Ceftazidime and Avibactam - linearity level -V**

**Table 11: Results of linearity level of Ceftazidime**

| S.No. | Linearity Level | Concentration | Area |
|---|---|---|---|
| 1 | I | 100ppm | 668934 |
| 2 | II | 200ppm | 956781 |
| 3 | III | 300ppm | 1313873 |
| 4 | IV | 400ppm | 1563458 |
| 5 | V | 500ppm | 1867084 |
| Correlation Coefficient | | | 0.997 |

**Table 12: Results of linearity level of Avibactam**

| S.No | Linearity Level | Concentration | Area |
|---|---|---|---|
| 1 | I | 1ppm | 66510 |
| 2 | II | 2ppm | 94701 |
| 3 | III | 3ppm | 124802 |
| 4 | IV | 4ppm | 152731 |
| 5 | V | 5ppm | 179732 |
| Correlation Coefficient | | | 0.999 |

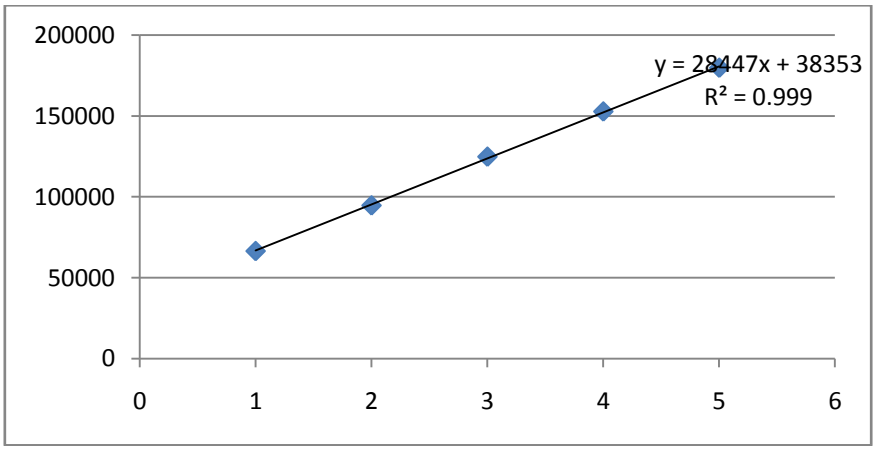



**Figure 33: Calibration graph for ceftazidime**

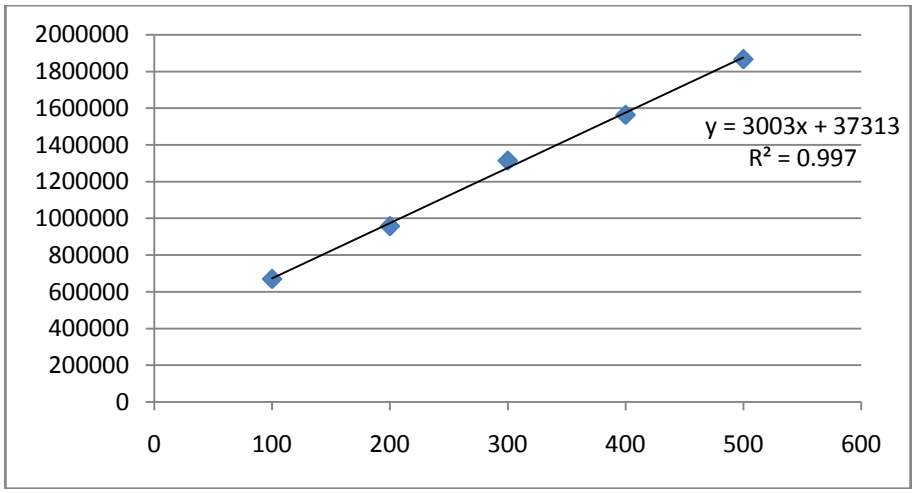



**Figure 34: Calibration graph for Avibactam**

**Table 13: Results of Linearity of Ceftazidime and Avibactam**

| **Parameters** | **Ceftazidime** | **Avibactam** |
|---|---|---|
| Slope (m) | 28447x | 3003x |
| Intercept (c) | 38353 | 37313 |
| Correlation coefficient ($R^2$) | 0.999 | 0.997 |

**Acceptance criteria:**
Correlation coefficient ($R^2$) should not be less than 0.999
The correlation coefficient obtained was 0.999 which is in the acceptance limit. The linearity was established in the range of 100% to 500% of ceftazidime and 1% to 5% of Avibactam.

**LIMIT OF DETECTION (LOD):**
The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio

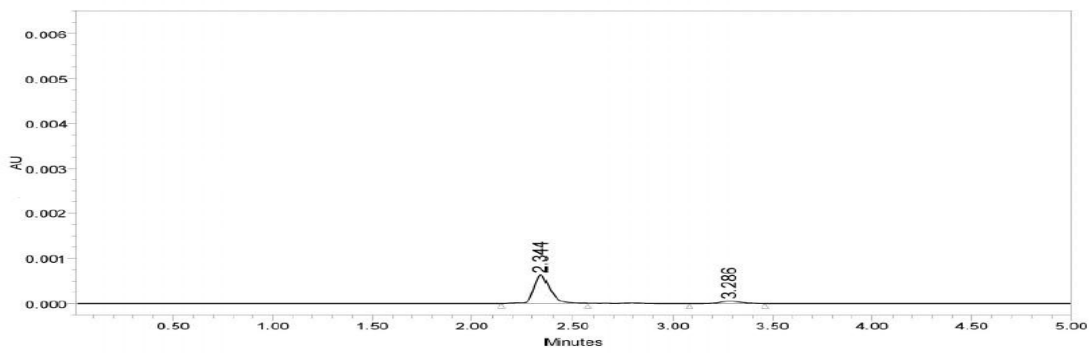



**Figure 35: Chromatogram of LOD for ceftazidime&Avibactam**

**Table 14: Results of LOD**

| **Drug name** | **Baseline noise(µV)** | **Signal obtained (µV)** | **S/N ratio** |
|---|---|---|---|
| Ceftazidime | 52 | 152 | 2.9 |
| Avibactam | 52 | 156 | 3 |

- Signal to noise ratio shall be 3 for LOD solution
- The result obtained is within the limit.

**LIMIT OF QUANTIFICATION (LOQ):**

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

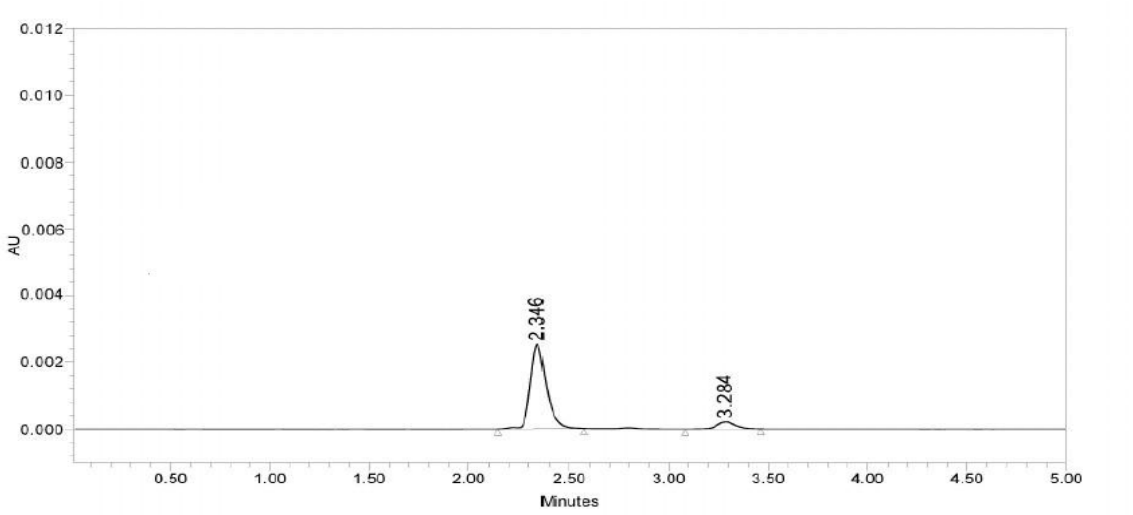



**Figure 36: Chromatogram of LOQ for ceftazidime&Avibactam**

**Table 15: Results of LOQ**

| Drug name | Baseline noise(µV) | Signal obtained (µV) | S/N ratio |
|---|---|---|---|
| Ceftazidime | 52 | 522 | 10.03 |
| Avibactam | 52 | 524 | 10.1 |

- Signal to noise ratio shall be 10 for LOQ solution
- The result obtained is within the limit.

**ROBUSTNESS:**

The standard and samples of ceftazidime and Avibactam were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

**Variation in Flow**

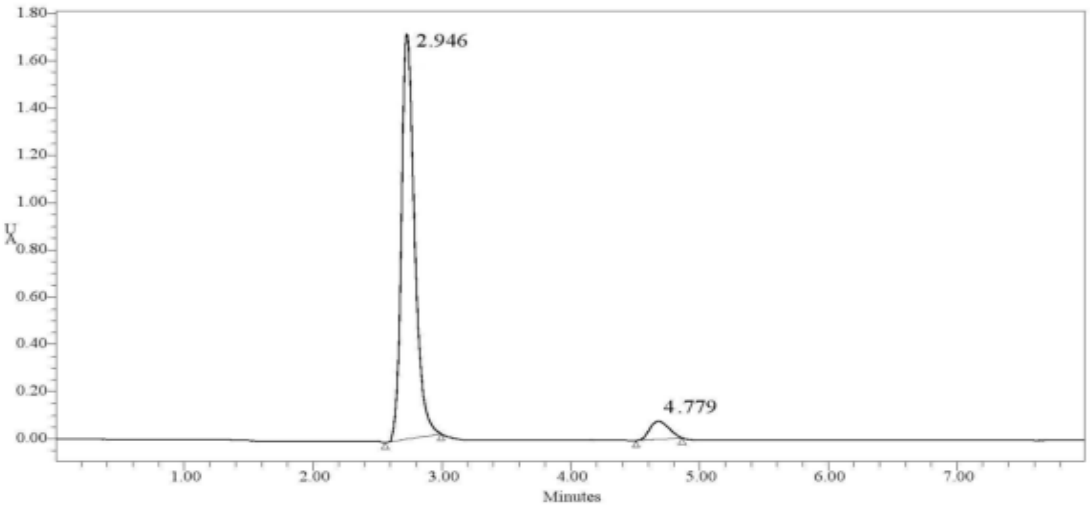



**Figure 37: Chromatogram of Ceftazidime and Avibactam (flow of 0.8ml/min)**

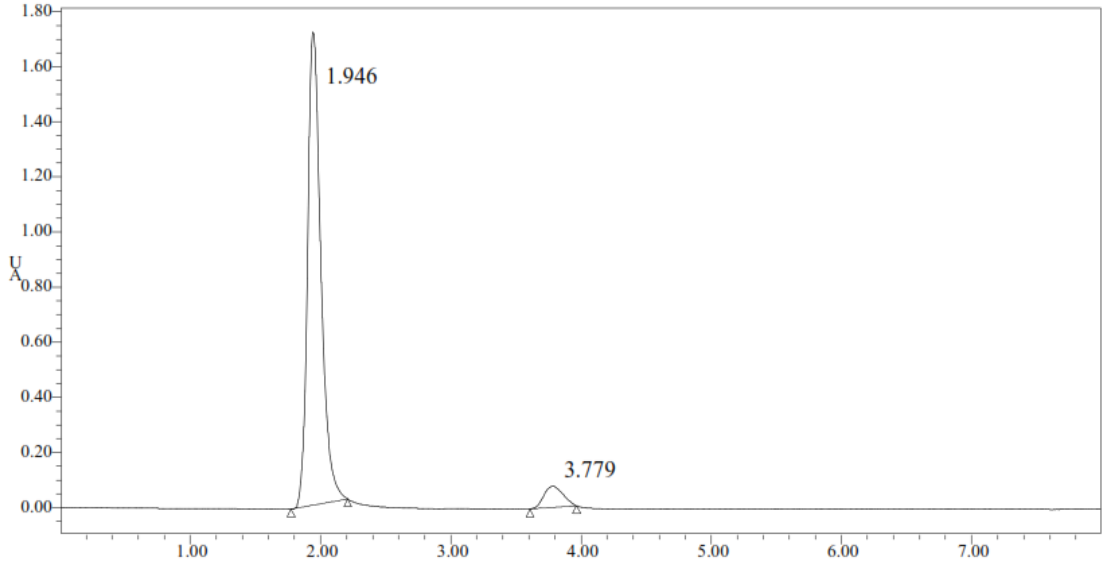



**Figure 38: Chromatogram of Ceftazidime and Avibactam (flow of 1.2ml/min)**

**Table 16: Results of Robustness (Flow Rate) data for ceftazidime**

| S. No | Flow Rate (ml/min) | System Suitability Results | |
|---|---|---|---|
| | | USP Plate Count | USP Tailing |
| 1 | 0.8 | 5339.9 | 1.4 |
| 2 | 1.0 | 4673.4 | 1.3 |
| 3 | 1.2 | 5216.0 | 1.4 |

**Table 17: Results of Robustness (flow rate) data for Avibactam**

| S. No | Flow Rate (ml/min) | System Suitability Results | |
|---|---|---|---|
| | | USP Plate Count | USP Tailing |
| 1 | 0.8 | 7063.3 | 1.3 |
| 2 | 1.0 | 6090.3 | 1.2 |
| 3 | 1.2 | 6998.0 | 1.3 |

**Variation of Mobile Phase Organic Composition:**

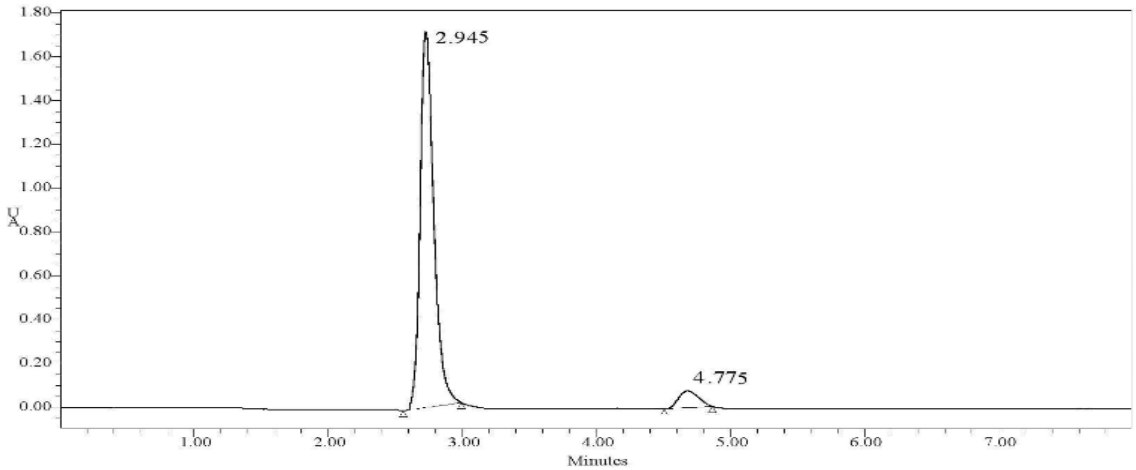



**Figure 39: Chromatogram for ceftazidime&Avibactam less organic composition**

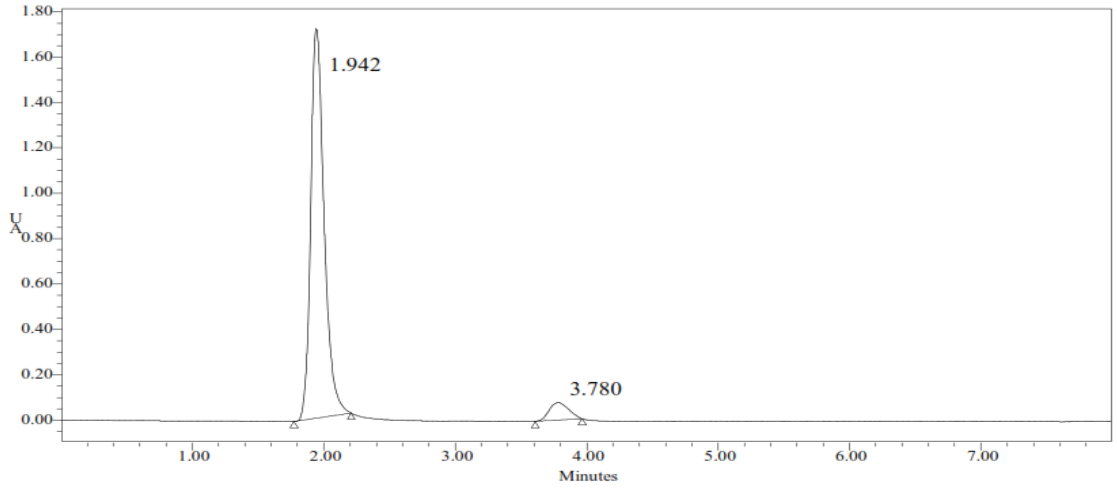



**Figure 40: Chromatogram Ceftazidime&Avibactam more organic composition**

**Table 18: Change in Organic Composition in the Mobile Phase for Ceftazidime**

| S.No | Changein Organic Composition in the Mobile Phase | System Suitability Results | |
|---|---|---|---|
| | | USP Plate Count | USP Tailing |
| 1 | 10% less | 4508.4 | 1.3 |
| 2 | *Actual | 4673.4 | 1.4 |
| 3 | 10% more | 4318.1 | 1.3 |

**Table 19: Change in Organic Composition in the Mobile Phase for Avibactam**

| S.No | Change in Organic Composition in the Mobile Phase | System Suitability Results | |
|---|---|---|---|
| | | USP Plate Count | USP Tailing |
| 1 | 10% less | 6387.7 | 1.2 |
| 2 | *Actual | 6090.3 | 1.2 |
| 3 | 10% more | 6232.5 | 1.2 |

**Acceptance criteria:**

Percentage RSD should be below 2.

- The %RSD obtained for change of flow rate, variation in mobile phase was found to be below 1, which is within the acceptance criteria.

## VI. CONCLUSION

High performance liquid chromatography is at present one of the most sophisticated tools of the analysis. The estimation of Ceftazidime and Avibactam was done by RP-HPLC. The Phosphate buffer was pH 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. Inertsil $C_{18}$ column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. the linearity range of Ceftazidime and Avibactam were found to be from 100-500 µg/ml of Ceftazidime and 1-5µg/ml of Avibactam. Linear regression coefficient was not more than 0.999.

The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of Ceftazidime and Avibactam. LOD and LOQ were found to be within limit.

The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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