RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR RELATED SUBSTANCE OF CEFIXIME ORAL SUSPENSION

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MASTER OF PHARMACY IN

PHARMACEUTICAL ANALYSIS

Submitted by

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"RP- HPLC METHOD DEVELPOMENT AND VALIDATION FOR RELATED SUBSTANCE OF "CEFIXIME ORAL SUSPENSION"** submitted by the student bearing **Reg. No:261630210** to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment for the award of degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL ANALYSIS** was evaluated by us during the examination held on…….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation **"RP- HPLC METHOD DEVELPOMENT AND VALIDATION FOR RELATED SUBSTANCE OF "CEFIXIME ORAL SUSPENSION"** submitted to The Tamilnadu Dr.M.G.R.Medical University, Chennai, was carried out by **Mr. VIKNESH S [Reg.No: 261630210],** for the Partial fulfillment of degree of **MASTER OF PHARMACY** in Department Of Pharmaceutical Analysis under direct supervision of **Dr.V.SEKAR, M.Pharm.Ph.D,** Head of Department Of Pharmaceutical Analysis, J.K.K.Nataraja College of Pharmacy, Komarapalayam, during the academic year 2017-2018.

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DECLARATION

The work presented in this dissertation entitled **"RP- HPLC METHOD DEVELPOMENT AND VALIDATION FOR RELATED SUBSTANCEOF "CEFIXIME ORAL SUSPENSION"** was carried out by me, under the direct supervision of **Dr.V.SEKAR,M.Pharm.Ph.D.,** Head of the department and professor, Department Of Pharmaceutical Analysis, J.K.K.Nataraja College of Pharmacy, Komarapalayam.I further declare that, this work is original and has not been submitted in part or full for the award of any other degree or diploma in any other university.

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Mr. VIKNESH S

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CONTENTS

LIST OF ABBREVIATIONS USED

ICH	-	International conference on Harmonization
USP	-	United states of Pharmacopoeia
λ	-	Lambda
µg/ml	-	Microgram per milliliter
ng /ml	-	Nanogram per milliliter
µl	-	Micro liter
ml	-	Milliliter
mM	-	Milli mole
nm	-	Nanometer
mm	-	Millimeter
%	-	Percentage
%RSD	-	Percentage of Relative standard Deviation
LOD	-	Limit of detection
LOQ	-	Limit of Quantitation
pH	-	Negative Logarithm of Hydrogen Ion
Rt	-	Retention time
S.D	-	Standard Deviation
RP-HPLC	-	Reverse phase –High performance liquid chromatography
min	-	Minute
ml /min	-	Milliliter / minute
v / v	-	Volume /Volume
ml /min	-	Millilitre /Minute

1. INTRODUCTION

Pharmaceutical Analysis is the branch of Chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in Bulk drug and Pharmaceutical preparations (Sharma B.K, 2000)

Pharmaceutical Analysis simply means analysis of a Pharmaceutical(s). It is generally known that a Pharmaceutical is a chemical entity of Therapeutic interest. A more appropriate term for a Pharmaceutical is Active Pharmaceutical Ingredient (API) or Active Ingredient. Even though the term Active Ingredient is more frequently used, the preferred term is Active Pharmaceutical Ingredient. To distinguish it from the formulated product or drug product, API is also called Drug substance. The drug product is prepared by formulating a drug substance with inert ingredients (excipients) to prepare a drug product that is suitable for administration to patients.

However, it should be recognized that there are situations where a drug substance can be administered after simple dissolution in a solvent such as water. Even in these situations, a suitable Pharmaceutical treatment has to be conducted to assure availability and other safety considerations.

It is well known in the Pharmaceutical industry that Pharmaceutical Analysts in Research and Development (R&D) play a very comprehensive role in new drug development and follow up activities to assure that a new drug product meets the established standards, is stable and continues to meet the purported quality throughout its shelf life. After the drug product is approved by regulatory authorities, assuring that all batches of drug product are made to the specified standards, utilization of approved ingredients and production methods becomes the responsibility of Pharmaceutical Analysts in the Quality Control (QC) or Quality Assurance (QA) department. The methods are generally developed in an analytical R&D department and transferred to QC or other departments, as needed. At times, they are transferred to other divisions located nationally or abroad or to outsourced

companies. By now it should be quite apparent that Pharmaceutical Analysts play a major role in assuring the identity, safety, efficacy, and quality of a Drug product. Safety and efficacy studies require that drug substance and drug product meet two critical requirements:

- Established Identity and Purity.
- Established Bioavailability / Dissolution **(Satinder A & Stephen S, 2001)**

Quality Assurance plays a central role in determining the safety and efficacy of medicines. Highly specific and sensitive analytical techniques hold the key to the design, development, standardization and quality control of medicinal products. They are equally important in pharmacokinetics and in drug metabolism studies, both of which are fundamental to the assessment of bioavailability and the duration of clinical response. However, modern concepts of quality differs and concerned not only with chemical purity, but also with those other characteristics of Pharmaceutical materials which may influence safety, efficacy, formulation and processing of medicines **(Beckett AH & Stenlake JB 1997)**.

1.1 INSTRUMENTAL ANALYSIS

The instrument is only one component of the total analysis. Often, it is necessary to use several instrumental techniques to obtain the information required to solve an analytical problem. Instrumental method may be used by analytical chemists to save time, to avoid chemical separation or to obtain increased accuracy.

Based on Principle Types of Chemical Instrumentation:

A) ***Spectrometric Techniques*****:**

- Atomic Spectrometry (Emission and Absorption)
- Electron Spin Resonance Spectroscopy
- Fluorescence and phosphorescence Spectrophotometry
- Infrared Spectrophotometry

- Nuclear Magnetic Resonance Spectroscopy
- Radiochemical Techniques including activation analysis
- Raman Spectroscopy
- Ultraviolet and visible Spectrophotometry
- X-Ray Spectroscopy

B) Electrochemical techniques

- Potentiometry
- Voltametry
- Stripping techniques
- Amperometric techniques
- Coulometry
- Electrogravimetry
- Conductance techniques.

C) *Chromatographic Techniques*:

- Gas Chromatography
- High performance Liquid Chromatography
- Thin Layer Chromatography

D) *Miscellaneous Techniques*:

- Kinetic Techniques
- Mass Spectrometry
- Thermal Analysis

D) *Hyphenated Techniques*:

- GC-MS (Gas Chromatography - Mass Spectrometry)
- ICP-MS (Inductivity Coupled Plasma - Mass Spectrometry)
- GC-IR (Gas Chromatography - Infrared Spectroscopy)
- MS-MS (Mass Spectrometry - Mass Spectrometry **(Willard H.H. *et al 1986)***

1.1. CHROMATOGRAPHY

Chromatography (from Greek: chroma, color and:"graphein" to write) is essentially a group of techniques for the separation of the compounds of mixtures by their continuous distribution between two phases, one of which is moving fast the other that depends on differential affinities of the solute between two immiscible phases, one of which will be fix with large surface area, while the other is fluid which moves through or over the surface of the fixed phase. **(Beckett AH & Stenlake JB 1997)**

Definitions for Chromatography:

1. Tswett gave the first definition of chromatography. Chromatography is a method in which the compounds of a mixture are separated on an adsorbent column in a flowing system.

2. Chromatography defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. **(Gurdeep R Chatwal & Sham K.Anand 2002)**

3. IUPAC: chromatography is a physical method of separation in which the compound to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction **(IUPAC, 1993)**

CLASSIFICATION OF CHROMATOGRAPHIC METHODS

(Gurdeep R. Chatwal & Sham K.Ananad 2002)

STATIONARY PHASE	MOBILE PHASE	NAME
SOLID	LIQUID	Plane Chromatography
		Paper Chromatography
		Thin layer Chromatography
		Adsorption Column Chromatography
		High Performance Liquid Chromatography
SOLID (Ion exchange resin)	LIQUID	Ion exchange Chromatography
SOLID	GAS	Gas-Solid Chromatography
SOLID MATRIX	LIQUID	Gel permeation Chromatography (Exclusion Chromatography)
LIQUID	GAS	Gas-Liquid Chromatography
LIQUID	LIQUID	Liquid-Liquid Chromatoraphy

1.3. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. Today it is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries.

HPLC has over the past decade become the method of choice for the analysis of a wide variety of compounds. Its main advantage over GC is that the analytes do not have to be volatile, so macromolecules are suitable for HPLC analysis.

PRINCIPLE:

HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of stationary phase. Separation of a mixture into its components depends on different degrees of retention of each component in the column. Since the compounds have different mobility's, they exit the column at different times; i.e., they have different retention times, Rt. The retention time is the time between injection and detection. There are numerous detectors which can be used in liquid chromatography. It is a device that senses the presence of components different from the liquid mobile phase and converts that information to an electrical signal.

Reversed phase HPLC

In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol. There will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent. Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of Vander Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. That means that now it is the polar molecules that will travel

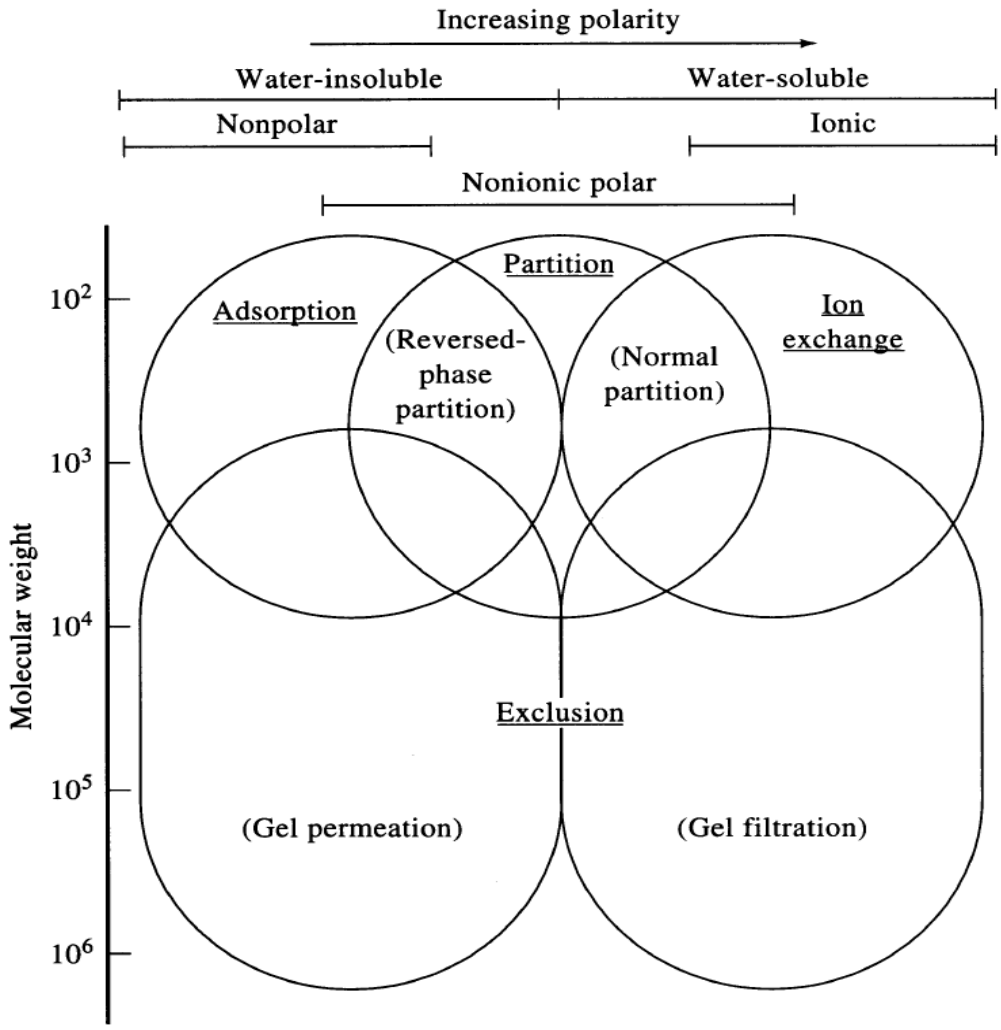
through the column more quickly. **(David C. Lee &Michael Webb, 2003)(Synder L.R &Kirkland J.J.,1997)**

The majority of the HPLC separations are done with Reversed phase separation, probably over 90%. In reversed phase separations organic molecules are separated based on their degree of hydrophobicity. There is a correlation between the degree of lipophylicity and retention in the column.

Types of HPLC techniques

- **Based on Principles of Separations**
  - Partition Chromatography
  - Adsorption (liquid-solid) Chromatography
  - Ion exchange Chromatography
  - Size exclusion Chromatography
- **Based on Modes of Chromatography**
  - Normal Phase Chromatography
  - Reverse Phase Chromatography
- **Based on Elution Techniques**
  - Isocratic Separation
  - Gradient Separation
- **Based on the Scale of Operation**
  - Analytical HPLC
  - Preparative HPLC

Flow chart: 1. Selection of HPLC methods depending upon Nature of samples

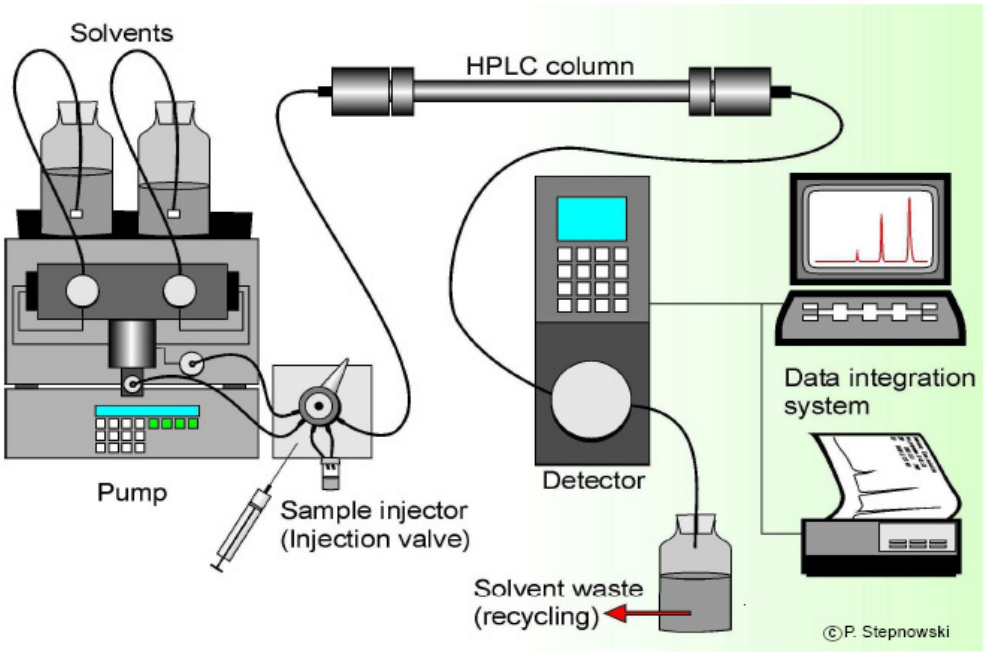


INSTRUMENTATION:

1. COLUMN:

HPLC columns are made of high quality Stainless steel, polished internally to a mirror finish. Standard analytical columns are 4-5 m internal diameter and 10-30 cm in length, shorter column (3-6 cm in length) containing a small particle size packing material (3 or 5 µm). **(Beckett AH& Stenlake J B 1997)**

Figure 1 : Shcematic representation of HPLC



Column packing:

Three forms of column packing material are available based on a rigid structure. These are

i. Microporous supports

ii. Pellicular supports

iii. Bonded phase supports (**Gurdeep R.Chatwal &Sham K.Anand,2002)**

2. MOBILE PHASE RESERVIOR :

The mobile phase reservior can be any clean, inert containers made up of stainless steel and glass. Precaution should be taken to present solvents spills in case of breakage of the reservoir and it should be placed in plastic container. Solvent bottles are available that are coated with a resin material that resist breaking. It usually contain 1 or 2 liter of solvent and it should have a cap that allows the tubing inlet line to pass through it. **(James W. Munson,2001)**

The choice of mobile to be used in any separation depend on the type of separation to be achieved. Isocratic separation may be made with a single solvent, or two or more solvents mixed in fixed proportion. Alternatively a gradient elution system may be used where the composition of the developing solvent is continuously changed by use of a suitable gradient programmer. All solvents for using HPLC systems must be specially purified since traces of impurities can affect the column and interfere with the detection system. It is also essential that all solvents are degassed before use other wise gassing tends to occur in most pumps. Gassing can alter column resolution and interfere with the continues monitoring of the column effluent. Degassing may be carried out in several way; by warming the solvents, by stirring it vigorously with a magnetic stir, subjecting it to a vacuum, ultrasonic vibrations or by bubbling helium gas through the solvent reservoir.(Gurdeep R.Chatwal & Sham K.Ananad,2002)

The following points should also be considered when choosing a Mobile phase:

1. It is essential to establish that the drug is stable in the Mobile phase for at least the duration of the analysis.

2. Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation, which can damage HPLC equipment.

3. The Mobile phase should have a pH between 2.5 to pH 7.0 to maximize the lifetime of the column.

4. Reduce cost and toxicity of the Mobile phase by using methanol instead of acetonitrile when possible.

5. Minimize the absorbance of buffer. Since trifluroacetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products with out chromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric acid, which does not absorb above 200 nm.

6. Use volatile Mobile phases when possible to facilitate collection of products and LC - MS analysis. Volatile Mobile phases include ammonium acetate, ammonium phosphate, and formic acid, acetic acid and trifluroacetic acid. Some caution is needed as these buffers absorb below 220 nm.

3. INJECTORS:

Injection ports are of two basic types

a. Those in which the sample is injected directly into the column

b. Those in which the sample is deposited before the column inlet and then swept by a vying action into the column by the mobile phase.

On –column injection involves the injection of the sample by means of a syringe through a septum into the center of the packing material. The column and the capacity of the packing material is typically 5-25µl for analytical column.

High- pressure syringes that can be used at pressure up to 650 atmospheres allow the injection of the sample while the mobile phase is flowing. While using Low- pressure syringes the flow must be stopped.

Modern injectors are based on injection valves which allow the sample at atmospheric pressure to be transferred to the high-pressure mobile phase immediately before the column inlet. With the injection in LOAD position, the sample is injected from a syringe through a needle port into the loop.

The valve lever is then turned through 60^{o} to the inject position and the sample is swept into the flowing mobile phase. An excess of sample is flushed through the loop in the LOAD position, the volume injected is the volume of the loop, which is typically 10-20µl for analytical separation. **(Beckett AH& Stenlake J B 1997)**

4. PUMPS:

The pumping system is one of the most important features of a HPLC system. There is a high resistance to solvent due to the narrow columns packed in small particles, high pressure are required to achieve satisfactory flow rate.

The requirements for an HPLC pumping system are several;

They include

a. The generation of pressures of up to 6000 psi (lbs/in^2)
b. Pulse- free output
c. Flow rates ranging from 0.1 to 10ml/min
d. Good flow control capacity
e. All materials in the pump should be chemically resistance to all solvents

These pumping system available which operate on the principle of constant pressure or constant displacement.

Constant pressure pumps produce a pulse less flow through the column, but any decrease in the permeability of the column will result in lower flow rates for which the pumps will not compensate. These pumps operate by the introduction of high pressure gas into the pump, and the gas in turn forces the solvent from the pump chamber in to the column. The intermediate solvent between the gas and the eluting solvent reduce the chances of dissolved gas directly enter in the eluting solvent and causing problems during the analysis.

Constant displacement pump maintain a constant flow rate through the column irrespective of changing conditions with in the column. One form of constant displacement pump is a motor-driven syringe type pump where a fixed volume of solvent is forced from the pump to the column by a piston driven by a motor. Such pumps, as well as providing uniform solvents flow rates, also yields a pulse less solvent flow which is important as certain in detectors are sensitive to change in solvent flow rate.

The reciprocating pump is most commonly used form of constant displacement pump. The piston is moved by a motorized crank and entry of solvent from the reservoir to the pump chamber and exit of solvent to the column is regulated by check valves. On the compression stroke solvent is forced from the pump chamber in to the column. During the return stroke the exit check valve closes and solvent is drawn in via entry valve to t e pump chamber, ready to be pumped on to the column on the next compression stroke. **(Gurdeep R.chatwal &Sham K. Anand,2002)**

5. DETECTORS:

The detector for an HPLC is the compound that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase on order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled (in most cases). There are many types of detectors that can be used with HPLC

Types of Detectors

1. Solute specific detectors (UV, visible, fluorescence, electrochemical, infra-red, radioactivity).
2. Bulk property detectors (refractive index, viscometer, conductivity).
3. Desolvation detectors (flame ionization etc.).
4. LC-MS detectors.
5. Reaction detectors.

Absorbance Detectors

Absorbance detectors is a typical, Z- shaped, flow through cell for absorbance measurements on eluent from chromatographic column. Volumes are limited to 1 to 10 µl and cell lengths to 2 to 10 mm, and the pressure not greater than 600 psi. Many Absorbance detectors are double-beam devices in which one beam passes through the eluent cell and the other through a filter to reduce the intensity.

Ultraviolet Absorbance Detectors

UV Absorbance Detectors are available in two types, UV Absorbance Detectors with Filter and with monochromators. Most HPLC manufactures offer detectors that consist of a scanning spectrophotometer with grating optics. Some are limited to UV radiation; others encompass both UV and Visible radiation. The most powerful UV Spectrophotometric detectors are diode - array instruments.

Refractive Index Detectors

RI Detectors are also called as Universal analyte detector. RI detectors have the significant advantage of responding to nearly all solutes. That is they are general detectors analogous to flame detectors in gas chromatography. In addition they are reliable and unaffected by flow rate. They are highly temperature sensitive and must be maintained at a constant temperature to a few thousands of a degree centigrade. They are not as sensitive as most other type of detectors and generally cannot be used with gradient elution.

Fluorescence Detectors

Excitation wavelength generates fluorescence emission. Analytes must contain a Flurophore group it reacts with the same group of the reagent. The inherent advantage of fluorescence methods is their high sensitivity. Results are dependent up on the separation condition. **(Gennaro A.R.Remigton, 2000)**

1.4. STEPS FOR ANALYTICAL DEVELOPMENT

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated.

1. Analyte standard characterization:

a) All information about the analyte i.e., physical and chemical properties, toxicity,

b) The standard analyte (100% purity) is obtained. Made an arrangement for the proper storage (refrigerator, desiccators and freezer).

c) When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.

d) Only those methods (MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements:

The goals of the analytical method that need to be developed are considered. The detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature search and prior methodology:

The information related to the analyte is surveyed for synthesis, physical and chemical Properties, solubility and relevant analytical methods. Books, periodicals and USP / NF, and publications are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient.

4. Choosing a method:

a) Using the information in the literatures, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.

b) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

5. Instrumental setup and initial studies:

a) The required instrumentation is setup Installation, operational and performance qualifications of instrumentation verified by using laboratory Standard Operating Procedures (SOP's).

b) Always new solvents, filters are used, for example, method development is never started, on a HPLC column that has been used earlier.

c) The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., Bulk drug), then it is possible to start work with the actual sample.

d) Analysis is done using analytical conditions described in the existing literature.

6. Optimization:

During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends.

7. Documentation of analytical figures of merit:

The originally determined analytical figures of merit Limit of Quantitation (LOQ), Limit of Detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of method development with actual samples:

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9. Determination of percent recovery of actual sample and demonstration of quantitative sample analysis:

a) Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average +/- standard deviation) from sample to sample and whether recovery has been optimized is determined. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty.

b) The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications.(Michael E & Schartz IS)

1.5. OPTIMIZATION OF CHROMATOGRAPHIC CONDITION

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that all the compounds are detected by more or less symmetrical peaks on the chromatogram. By a slight change of the mobile phase composition, the shifting of the peaks can be expected. From a few experimental measurements, 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 run time. **(Munson J.W,1994)**

The peak resolution can be increased by using a more efficient column with higher theoretical plate number, N.

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

- Resolution (R_s),
- Capacity factor (k'),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry factor (As).

i) Resolution (R_s)

The resolution, R_s, of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 1.5. It is calculated by using the formula,

$$R_s = \frac{Rt_2 - Rt_1}{0.5(W_1 + W_2)}$$

Where, Rt_1 and Rt_2 are the retention times of components 1 and 2 and

W_1 and W_2 are peak widths of components 1 and 2.

ii) Capacity factor (k')

Capacity factor, k', is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column or TLC plate during an isocratic separation. The ideal value of k' ranges from 2-10. Capacity factor can be determined by using the formula,

$$k' = \frac{V_1 - V_0}{V_0} \times S$$

Where, V_1 = retention volume at the apex of the peak (solute) and

V_0 = void volume of the system.

The values of k'of individual band increase or decrease with changes in solvent strength. In reverse phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water / organic mobile phase. Typically an increase in percentage of the organic phase by 10 % by volume will decrease k' of the bands by a factor of 2-3.

iii) Selectivity (α)

The selectivity (or separation factor), α, is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the formula,

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak respectively.

iv) Column efficiency (N)

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 2000 - 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16\frac{Rt^2}{W^2},$$

Where, Rt is the retention time and W is the peak width.

v) Peak Asymmetry factor (As)

Peak asymmetry factor, can be used as a criterion of column performance. The peak half width, b, of a peak at 10 % of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor.

$$As = \frac{b}{a}$$

For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable. **(Jeffery G.H *et al , 2003)***

1.6. VALIDATION

The word "validation" means "Assessment" of validity or action of validity or action of providing effectiveness'. Validation is, of course, a basic requirement to ensure quality and reliability of the results for all analytical applications. However, in comparison with Analytical Chemistry, in Pharmaceutical Analysis, some special aspects and conditions exist that need to be taken into consideration. Validation of an analytical method is the process by which it is established by laboratory studies,

that the performance characteristics of the method meet the requirements for the intended analytical applications.

Definitions:

Validation is a systematic approach to gathering and analyzing sufficient data which will give reasonable assurance (documented evidence), based upon scientific judgment, that a process, when operating within specified parameters, will consistently produce results within predetermined specifications.

Validation is defined as follows by different agencies:

European Committee (EC):

Action of providing in accordance with the principles of Good Manufacturing Practice (GMP) that any procedure, process, equipment, material, activity or system actually leads to the expected results. In brief validation is a key process for effective Quality Assurance

Food and Drug Administration (FDA):

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

World Health Organization (WHO):

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

History:

Since the mid-1970s validation has become an increasingly dominant influence in the manufacturer and quality assurance of pharmaceutical products. In 1976 the FDA proposed a whole set of current GMP regulations which were revised several times.

Objective of the Validation

There are two important reasons for validating assays in the Pharmaceutical Industry.

- The first, and by for the most important, is that assay validation is an integral part of the quality control system.

- The second is that current Good Manufacturing Practice (GMP) regulation requires assay validation. In industry it would be difficult to confirm that the product being manufactured is uniform and that meet the standards set to assure fitness for use. The varying nature of the differences between the analytical development laboratory and quality control laboratory is a good reason for validation program.(USP 1985) (Joachim Ermer& Miller2005)

ANALYTICAL METHOD VALIDATION

Method Validation is the process of proving that an analytical method is acceptable for its intended purpose. Methods need to be validated or revalidated- Before their introduction into routine use, whenever the conditions change for which the method has been validated, whenever the method is changed and the change is outside the original scope of the method.

- United States Pharmacopoeia (USP).

- Food and Drug Administration (FDA).

- World Health Organization (WHO).

- International Conference on Harmonization (ICH).

These guidelines provide a framework for performing Validation. In general, methods for routine analysis, standardization or regulatory submission must include studies on specificity, linearity, accuracy, precision, range, limit of detection, limit of Quantitation and robustness.

In the early stages of drug development, it is usually not necessary to perform all of the various validation studies. Many researchers focus on specificity, linearity, accuracy, and precision studies for drugs in the preclinical through Phase II (preliminary efficacy) stages. The remaining studies are performed when the drug reaches the Phase III (efficacy) stage of development and has a higher probability of becoming a marketed product. The process of validating a method cannot be separated from the actual development of the method conditions, because the developer will not know whether the method conditions are acceptable until validation studies are performed. The development and validation of a new analytical method may therefore be an iterative process. Results of validation studies may indicate that a change in the procedure is necessary, which may then require revalidation.

During each validation study, key method parameters are determined and then used for all subsequent validation steps. To minimize repetitious studies and ensure that the validation data they are generated under conditions equivalent to the final procedure. **(Mark JG.)**

Benefits of Method Validation:

A fully validated process may require less in-process control and end product testing. It deepens the understanding of processes, decrease the risks of processing problems, and thus assure the smooth running of the process**.**(WHO 1999)

Validation Parameters of Analytical Method:

According to ICH guidelines, typical analytical performance characteristics that should be considered in the validation of the types of methods are

Typical Validation Characteristics which should be considered are:

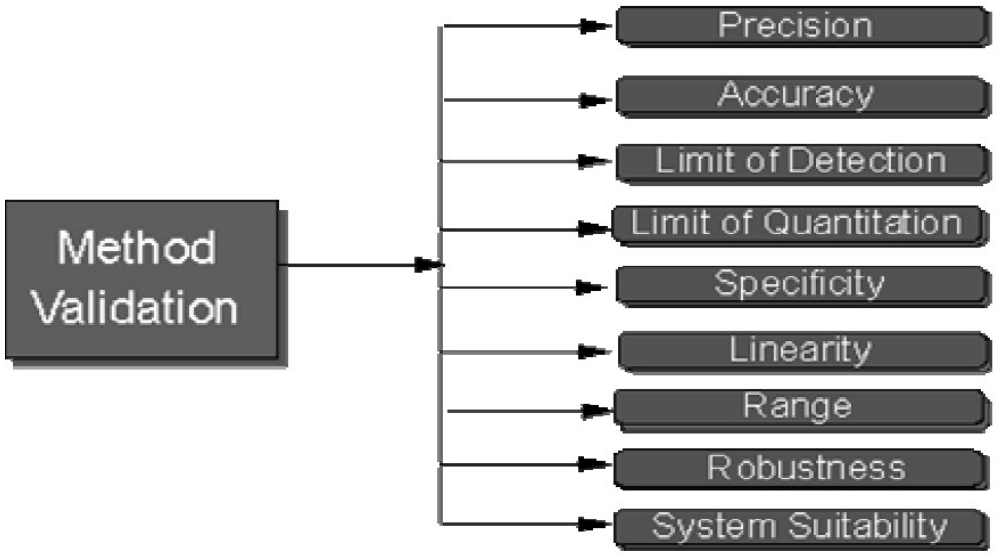


Figure 5: The USP and ICH Method Validation Parameter

1. 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.

The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels the specified range (i.e., three concentrations and three replicates of each concentration). Accuracy was tested (% Recovery and % RSD of individual measurements) by analyzing samples at least in triplicate, at each level (80,100 and 120 % of label claim) is recommended. For each determination fresh samples were prepared and assay value is calculated. Recovery was calculated from following regression equation obtained in linearity study.

The % recovery was calculated using the formula,

$$\%\,\mathrm{Recovery} = \frac{(a+b)-a}{bX100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

2. Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. The ICH documents recommend the repeatability should be assessed using a minimum of nine determinations covering specified range of procedure.

2.1) Repeatability:

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

2.2) Intermediate Precision:

Intermediate precision expresses with in laboratories variations: different days, different analyst and different equipment.

2.3) Reproducibility:

When the procedure is carried out by different analyst in different laboratories using different equipment, regents and laboratories setting reproducibility was determined by measuring repeatability and intermediate precision. Reproducibility is assessed by means of an inter-laboratory trial.

3. Specificity:

An ICH document defines 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. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

The definition has the following implications:

Identification test:

To ensure identity of an analyte.

Purity test:

To ensure that all the analytical procedures performed allow an accurate statement of the content of impurity of the content of impurity of an analyte i.e. related substances test, heavy metals, residual solvents etc.

Assay:

To provide an exact result, this allows an accurate statement on the content or potency of the analyte in a sample.

4. Limit of Detection (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. The detection limit is usually expressed as the concentration of analyte (percentage parts per million) in the sample.

Determination of Detection Limit:

For instrumental and non-instrumental methods detection limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected.

$$LOD = 3.3\ \sigma / S$$

Where

σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte)

5. Limit of Quantitation (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. The Quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. Quantification limit is expressed as the concentration of analyte (e.g. - % ppms) in the sample.

Determination of Quantification Limit

For instrumental and non- instrumental methods, the Quantitation limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

$$LOQ = 10\,\sigma / S$$

Where

σ = the standard deviation of the slope

S = the slope of the calibration curve (of the analyte)

5.1) Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

***5.2)* Based on the Calibration Curve**

A specific calibration curve should be studied using samples, containing an analyte in the range of LOQ. The residuals SD of regression line or the SD of intercepts of regression lines may be used as the SD. The quantitative limit is a parameter of quantitative assay for low levels of compounds in sample matrices, and is use particularly for the determination of impurities or degradation products.

6. 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.The linearity is determined from 60% of the ICH reporting

level to 140 % of the proposed shelf life specifications of the related substance as a minimum.

7. Range:

The range of an analytical procedure is the interval between the upper and lower of analyte, which is studied.

The range of an analytical procedure was the concentration interval over which acceptable accuracy, precision and linearity were obtained. In practice, the range was determined using data from the linearity and accuracy studies. Assuming that acceptable linearity and accuracy (recovery) results were obtained as described earlier. The only remaining factor to be evaluated was precision. To confirm the 'range' of any analytical procedure, linearity studies alone are not sufficient, and accuracy at each concentration (minimum three concentration levels covering lower and upper levels) should be proved.

8. Ruggedness:

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, different instruments etc, normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method. Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst. Degree of representative of test results is then determined as a function of the assay variable.

9. Robustness:

Robustness of an analytical method is measure of its capacity to remain unaffected small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. (www.waters.com, USP specification) (ICH Guidelines 1996)

Table C: Acceptance criteria of validation for HPLC

S.No.	Characteristics	Acceptance criteria
1.	Accuracy	Recovery 98-102% with 80,90,100,120 spiked sample
2.	Precision	-
a)	Repeatability	RSD < 2
b)	Intermediate precision	RSD < 2
3.	Specificity / Selectivity	No interference
4.	Detection limit	S/N > 2 or 3
5.	Quantitation limit	S/N > 10
6.	Linearity	$r^2 > 0.999$
7.	Range	80 - 120%
8.	Stability	>24hr or < 8hr

2. LITERATURE REVIEW

Systematic literature survey is the main basis for the planning of any scientific work and due to the same reasons here the review of literature regarding estimation of cefixime in oral suspension dosage formulation

1. Andrew J. Falkowski, Zee M. Look, Hideyo Nouguchi, B. Michael Silber. Determination of cefixime in biological samples by RP-HPLC. Journal of Chromatography 1987; 422: page no:145-52 The Cefixime Trihydrate and Sulbactam Sodium belong to a group of Anti-bacterial drugs. A Simple, Rapid, Specific and economic Reverse phase High Performance Liquid Chromatographic (RP- HPLC) method has been developed for assaying both the drugs in combinational dosage form. Method involves elution of Cefixime Trihydrate and Sulbactam Sodium in Hyper ODS2, Column C18, 150 x 4.6 mm (5 µm) using mobile phase composition of a mixture of 45 ml Acetonitrile and 55 ml of water, pH 6.5 adjusted with OPA at flow rate 1ml/min and analytes were monitored at 254 nm.Method has been validated according to ICH (International Conference on Harmonization) Guideline.

2. Dhoka M, Gawande V, Joshi P, Simultaneous Estimation of Cefixime Trihydrate and Erdosteine in Pharmaceutical Dosage form by using reveres phase – High Performance Liquid Chromatography, International Journal of ChemTech Research, Jan-Mar 2010, Vol.2, No.1, page no:79-87. simple, precise, and sensitive high-performance liquid chromatographic method was developed and validated for the simultaneous determination of potassium clavulanate and cefixime in synthetic mixture form. The analytes were separated on a C18 column by using 0.03 M disodium hydrogen phosphate buffer (pH 6.5)methanol (84 + 16, v/v) as the mobile phase with detection at 220 nm. The method exhibited high sensitivity and good linearity in the concentration ranges of 12.562.5 and 200 mg/mL for potassium clavulanate and cefixime, respectively. The total run time for the 2 components was <8 min, and the average recovery was >101.5 with a relative standard deviation of <1.0. The proposed method was validated according to guidelines of the International Conference on Harmonization by evaluation of linearity, recovery,

selectivity, robustness, limits of detection and quantitation, and within- and between-day precision. The results obtained for the synthetic mixture show that the method is highly precise and accurate for the simultaneous determination of potassium clavulanate and cefixime.

3. Eric-Jovanovi S, Agbaba D, Zivanov-Stakic D, Vladimirov S. HPTLC determination of cephalosporins in dosage forms. Journal of Pharmaceutical and Biomedical Analysis 1998; 18: page no 893-98. A simple, precise, accurate, and sensitive RP-HPLC method for simultaneous determination of cefixime trihydrate and dicloxacillin sodium in combined tablet dosage form was developed and validated. Chromatographic separation of the two drugs was performed on a Purospher BDS C18 column (25 cm 4.6 mm id, 5 m particle size). The mobile phase methanol0.01 M phosphate buffer (75 + 25, v/v), adjusted to pH 3 with glacial acetic acid, was delivered at a flow rate of 1.0 mL/min. Detection was performed at 227 nm. Separation was completed within 10 min. Calibration curves were linear with R^2 between 0.99 to 1.0 over a concentration range of 210 g/mL for cefixime trihydrate and 525 /mL for dicloxacillin sodium. The RSD for intraday and interday precision was <2.0.

4. Global Quality Guideline. Validation of Analytical Procedures. 2002; Number: G-6.9, Version: 1.0.. page no 620-667 A simple and accurate method to determine tadalafil (TAD) in pure powder and tablet dosage form was developed and validated using HPLC. The separation was achieved on an Xterra RP18 column (150 4.6 mm id, 3.5 m) in the isocratic mode using bufferacetonitrile (70 + 30, v/v), adjusted to pH 7.00 0.05 with triethylamine as the mobile phase at a flow rate of 1.0 mL/min. The photodiode array detector was set at 225 nm. Quantification was achieved over the concentration range of 50.7152.10 g/mL with mean recovery of 100.26 0.75. The method was validated and found to be simple, accurate, precise, and specific. The method was successfully applied for the determination of TAD in pure powder and tablet dosage form without interference from common excipients or degradation products.

5. Khan U, Sharif S, Ashfaq M, Asghar N, Simultaneous Determination of Potassium Clavulanate and Cefixime in Synthetic Mixtures by High Performance

Liquid Chromatography, Journal of AOAC International, July 1 2008, Vol 91, page no 744-749 Two sensitive and reproducible methods are described for the quantitative determination for the simultaneous estimation of cefixime trihydrate and ambroxol hydrochloride. The first method was based on HPTLC followed by densitometric measurements of their spots at 254 nm. The separation was on HPTLC aluminium sheets of silica gel 60 F254 using acetonitrile: methanol: triethylamine (8.2:1:0.8, v/v/v) as mobile phase. The linear regression analysis was used for the regression line in the range of 200 - 1000 ng spot-1 for cefixime and ambroxol, respectively. This system was found to give compact spots for cefixime and ambroxol, after development. The second method was based on HPLC separation of the two drugs on the column [C18 (5 µ, 25 cm×4.6 mm, i.d.)] at ambient temperature using a mobile phase consisting of acetonitrile: methanol (50:50, v/v). Quantitation was achieved with UV detection at 254 nm based on peak area with linear calibration curves at concentration ranges 4 - 18 and 4 - 28 µg mL-1 for cefixime and ambroxol, respectively. Both methods have been successively applied to pharmaceutical formulation. No chromatographic interference from the tablet excipients was found. Both methods were validated in terms of precision, robustness, recovery and limits of detection and quantitation

6. Kumudhavalli M, Sahu S, Abhiteja K, Jayakar B, Development and Validation of RP-HPLC method for simultaneous determination of Cefixime and Potassium Clavulanate in Tablet Dosage Form, International Journal of Pharma Recent Research, June-September 2010, Vol No 2. page no 320-345, page no 2A simple and sensitive reversed phase High Performance Liquid Chromatographic method has been developed and validated for the simultaneous analysis of the Cefixime trihydrate (CEF) and Linezolid (LIN) in tablet dosage form. The separation was carried out using mobile phase consisting of buffer and methanol with pH 2.5 in the ratio of 70:30, v/v. The column used was ACE 5 C18, (150 mm x 4.6 mm i.d., 5 µm) with flow rate 1.2 ml/min using PDA detection at 250 nm. The method was linear over a concentration range of 23.33 – 40 µg/ml and 70 – 120 µg/ml for CEF and LIN, respectively.

3. AIM AND OBJECTIVE OF WORK

The drug analysis plays an important role in the development of drugs, their manufacture and the therapeutic use. Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw materials used and the final product obtained meets the required specification. The number of drugs and drug formulations introduced in to the market has been increasing. These drugs or formulation may be either in the new entities in the market or partial structural modification of the existing drugs

The single component dosage form proves to be effective due to the mode of action on the body. The dosage forms including the presence of drug entities possess considerable challenge to the analytical chemist during the development of related substance procedure.

For the present study of Cefixime was selected. The extensive literature survey carried out and revealed that there is one method reported for the Related substance of cefixime oral suspension. Hence an attempt was made to develop a specific, precise, accurate, linear, simple, rapid, validated and cost effective RP-HPLC method for the study of these drugs in the dosage forms.

4. PLAN OF WORK

To develop and validate an effective RP – HPLC method for the estimation of *Cefixime* in bulk and its pharmaceutical dosage forms.

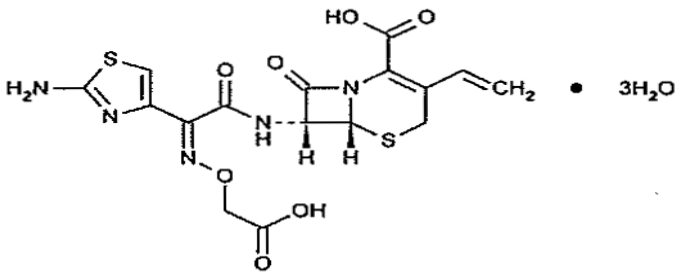
So ,the plan of work for the designed study was as follows:

- Gathering physical chemical properties of drug
- From the UV- analysis ,selection of λ max
- Selection of chromatographic condition
    - Selection of stationary phase
    - Selection of mobile phase
    - Selection of flow rate
    - Selection of Initial separation condition
- Optimization of chromatographic condition
- Validation of proposed method
- Applying developed method to the marketed formulation.
- Summarize methodology, finalize documentation.

5. DRUG PROFILE

Cefixime Trihydrate

Structure **:**



Chemical name **:** (6R,7R)-7-{[2-(2-amino-1,3-thiazol-4-yl)-2-(carboxy methoxyimino)acetyl]amino}-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

Description **:** White to light yellow, crystalline power

Molecular formula **:** $C_{16}H_{15}N_5O_7S_2 \cdot 3H_2O$

Molecular mass **:** 507.50 g/mol

Bioavailability **:** 40-50%

Half- life **:** 3 - 4 hours

Category : Antibiotic

M.O.A **:** Cefixime binds to specific penicelline binding protein (PBPs) located iniside the bacterial cell wall causing the inhibition of the third and last stage of bacterial cell wall synthesis, whiche final transpeptiation step of the peptidoglycan synthesis in the bacterial cell wall. Thus inhibiting biosynthesis and arresting cell wall assembly resulting in bacterial cell death.

Adverse reaction : Diarrhoea & abdominal pain
- Headache
- Nausea
- Allergic reaction
- It is not recommended history of severe penicilline allergy
- Urticaria
- Dizziness,
- Loose stools

Dose : 100 mg/5mL

Pharmacokinetics

Cefixime is an orally active cephalosporin antibiotic which has in-vitro bactericidal against a wide variety of Gram-positive and Gram-negative organisms including Streptococcus pneumonia, Streptococcus pyrogens, Escherichia coli, Proteus mirabilis, Klebsiella species, Haemophilus influenzae, (beta-lactamase positive and negative), Moraxella (Branhamella) catarrhalis (beta-lactamase positive and negative). Cefixime is stable in the presence of beta-lactamase enzymes.

Most strains of enterococci (Streptococcus faecalis, group D Streptococcus) and staphylococci (including coagulase positive and negative strains andmethicillin resistant strains) are resistant to cefixime. In addition, most strains of Enterobacter and Pseudomonas, bacteroides fragilis, Listeria monocytogenes and Clostridia are resistant to cefixime.

Pharmacodynamics

Cefixime an a antibiotic, is a third generation of cephalosporin group cefixme is highly stable in the presence of beta-lactamase enzymes its inhibition mucopeptide synthesis in the bacterial cell wall

Uses : common cold, flu

6. MATERIALS AND INSTRUMENTS

Instruments used:

- System : HPLC Agilent-2695 infinity
- Pump : I80 (LC – 10 AT Vp series)
- Detector : UV/Visible E2469
- Column : Novapak C $_{18}$ column. (150mm x 3.9 mm, 4 µ)
- pH meter : Elico
- Digital balance : Sartorious BSA224S-CW
- Sonicator : PCI Analytics

Reagents and Chemicals

- Acetonitrile : HPLC grade(Merck)
- Water : HPLC grade(MilliQ)
- Ortho phosphoric acid : AR grade(Merck)
- Potassium dihydrogen phosphate : AR grade(Merck)
- Tetra butyl ammonium hydroxide : AR grade(Merck)

  solution (40% in water)

Reference Standards and sample

1. Cefixime purity : 89.0 %
2. Oral suspension Brand Used : Supraxime oral suspension
3. Label claim of Cefixime : 100 mg/5mL

7. METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

SOLUBILITY

According to literature, Cefixime Soluble in methanol and in propylene glycol; slightly soluble in alcohol, in acetone, and in glycerin; very slightly soluble in 70% sorbitol and in octanol; practically in soluble in ether, in ethyl acetate, in hexane, and in water.

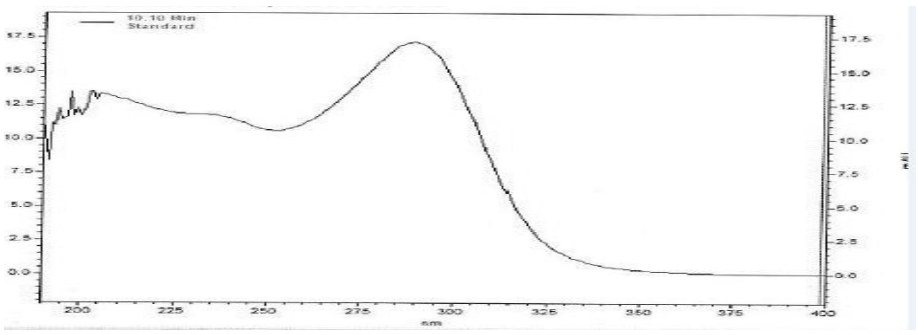
SELECTION OF CHROMATOGRAPHIC CONDITION

The drugs selected in the present study are polar in nature and hence reversed phase or ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability.

SELECTION OF WAVELENGTH (λ max)

In setting up the conditions for the development of the related substances method, the choice of detection wavelength was based on the scanned absorption for Cefixime. The spectrum was scanned over the range of 190 – 400nm and was obtained by measuring the absorption of 1.0 mg/ml solution of Cefixime in methanol and water prepared from stock solution. The spectrum was obtained by using HPLC. λ_{max} of cefixime was 254. Hence for estimation 254 nm was selected. It shown in *(fig no :1*)

Spectrum of cefixime figure no: 1



7.1. METHOD DEVELOPMENT TRIALS

Trial –1

Preparation of Buffer Mobile Phase A:

Mix 33 mL of Tetra butyl ammonium hydroxide solution (40% in water) in 1000 ml of water. Adjust the pH to 6.5 using orthophosphoric acid. Filter through 0.45µ membrane filter.

Preparation of Mobile phase B: Acetonitrile.

Diluent : Methanol and water

Mobile phase Composition : Mobile phase A : Mobile phase B

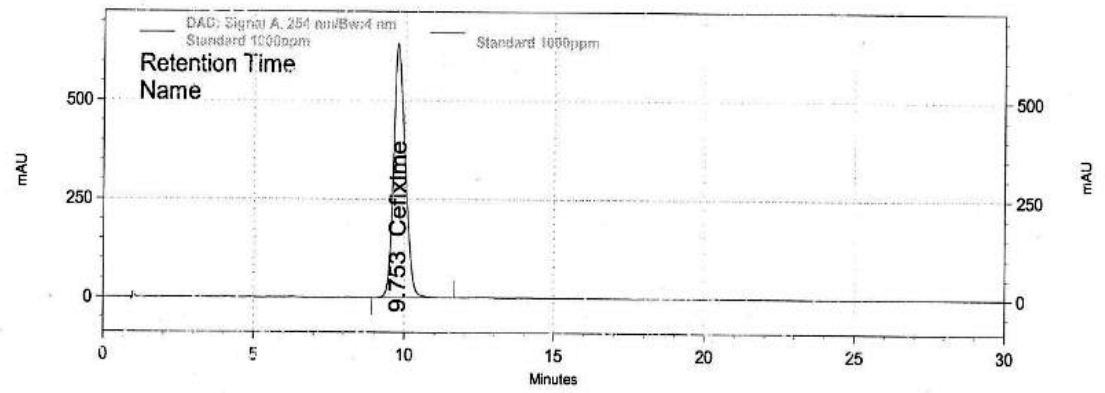
80 : 20

The trail 1 was performed in the mobile phase of mobile phase A and mobile phase B in the ratio of 80 : 20 with the flow rate of 1 ml/min by using the C_{18} Novapak 150x3.9mm, 4 µ column and column temperature 40°C

Result: While injecting the above chromatographic condition, Analyte peaks RT was found identified separately.

Chromatogram no : 1

Trial : 1



Trial –2

Preparation of Buffer Mobile Phase A:

Mix 33 mL of Tetra butyl ammonium hydroxide solution (40% in water) in 1000 ml of water. Adjust the pH to 6.5 using orthophosphoric acid. Filter through 0.45 µ membrane filter.

Preparation of Mobile phase B: Acetonitrile.

Diluent : Methanol and water

Mobile phase Composition : Mobile phase A : Mobile phase B

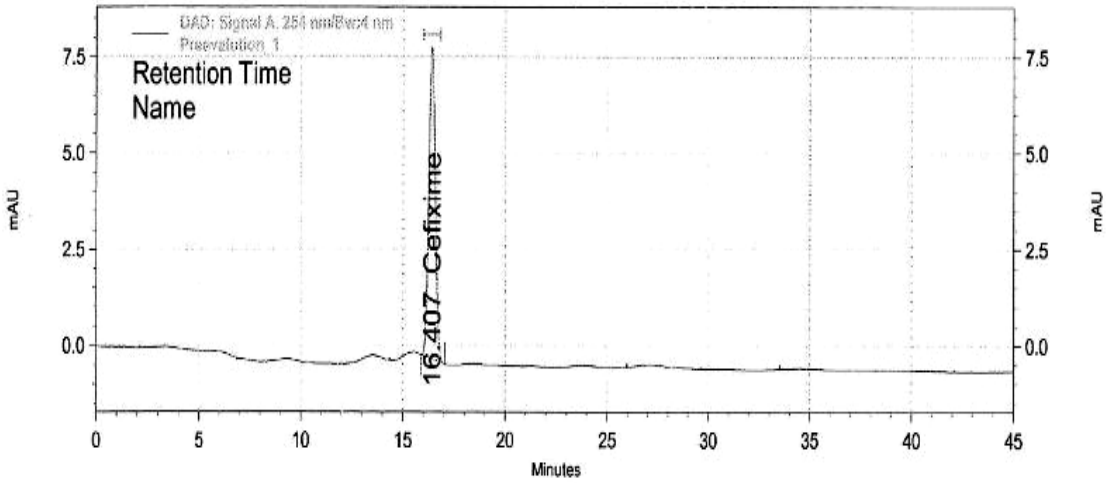
85 : 15

The trail 2 was performed in the mobile phase of Mobile phase A and Mobile phase B in the ratio of 85 : 15 with the flow rate 1.0 ml/min C_{18} Novapak 150x3.9mm, 4um column and column temperature 40°C

Result: While injecting the above chromatographic condition, the impurities was merged with analyte peak , Diluent and mobile phase ratio should not be shoutable,

Chromatogram no : 2

Trial : 2



TRIAL - 3

Preparation of Buffer Mobile Phase A:

Mix 33 mL of Tetra butyl ammonium hydroxide solution (40% in water) in 1000 ml of water. Adjust the pH to 6.5 using orthophosphoric acid. Filter through 0.45 µ membrane filter.

Preparation of Mobile phase B: Acetonitrile.

Diluent : potassium dihydrogen phosphate 0.1M adjust the PH-6.5 using with disodium Hyrogen phosphate 0.1M

Mobile phase Composition **:** Mobile phase A : Mobile phase B

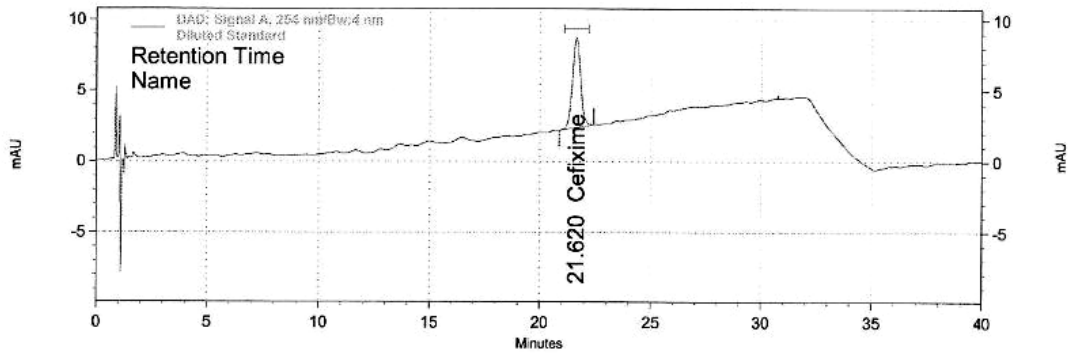
90 : 10

The trail 3 was performed in the mobile phase of Mobile phase A and Mobile phase B in the ratio of 90:10 with the flow rate of 1.0 ml/min C_{18} Novapak 150 x 3.9mm, 4um column and column temperature 40°C

Result: While injecting the above chromatographic condition, the impurities was merged with analyte peak so it preferable to gradient programme

Chromatogram no : 3

Trial : 3



TRIAL –4

Preparation of Buffer Mobile Phase A:

Mix 33 mL of Tetra butyl ammonium hydroxide solution (40% in water) in 1000 ml of water. Adjust the pH to 6.5 using orthophosphoric acid. Filter through 0.45 µ membrane filter.

Mobile phase B: Acetonitrile.

Diluent : potassium dihydrogen phosphate 0.1M adjust the PH-7.0 using with disodium Hyrogen phosphate 0.1M

Chromatographic condition the flow rate of 1.0 ml/min C_{18} Novapak 150 x 3.9mm, 4um column and column temperature 40°C

Gradient program

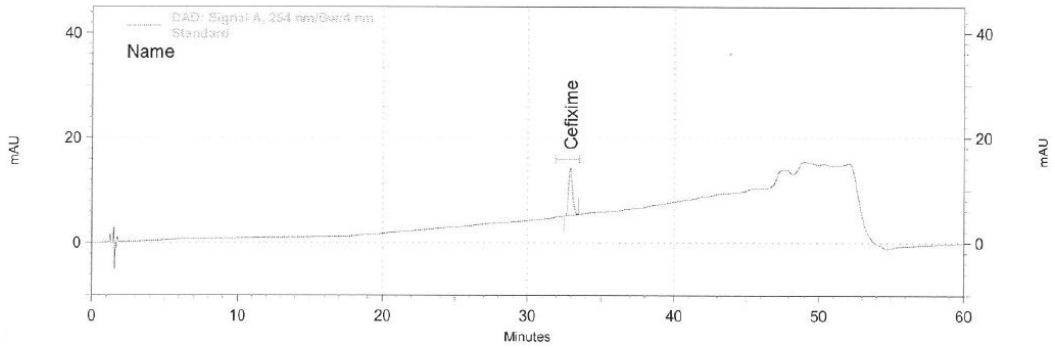
Time	Mobile phase A	Mobile phase B
0	90	10
15	90	10
50	65	35
52	90	10
60	90	10

Result:

The cefixime was separated with impurity peak but very less value resolution should be produced so need to slightely changes to be gradient program

Chromatogram no : 4

Trial : 4



TRIAL –5

Preparation of Buffer Mobile Phase A:

Mix 33 mL of Tetra butyl ammonium hydroxide solution (40% in water) in 1000 ml of water. Adjust the pH to 6.5 using orthophosphoric acid. Filter through 0.45 µ membrane filter.

Mobile phase B: Acetonitrile.

Diluent : potassium dihydrogen phosphate 0.1M adjust the pH-7.0 using with disodium Hyrogen phosphate 0.1M,

Gradient program

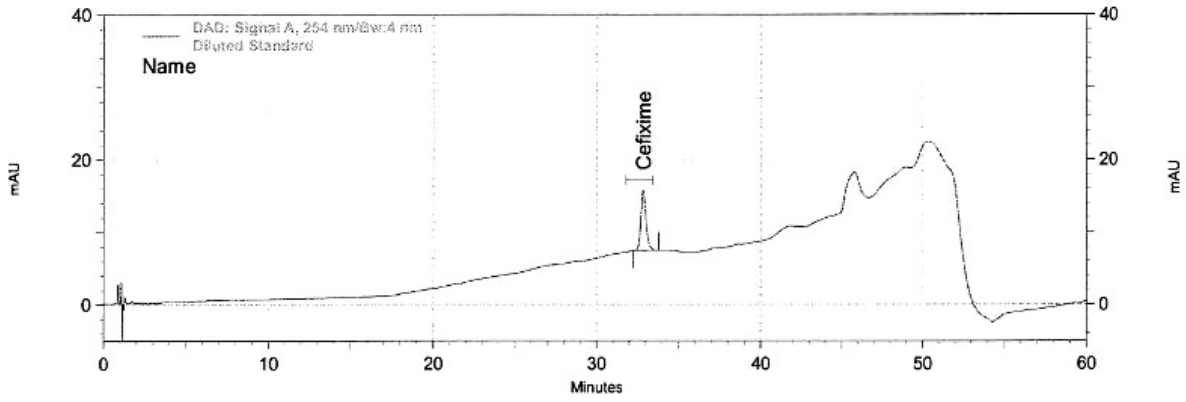
Time	Mobile phase A	Mobile phase B
0	90	10
15	90	10
50	70	30
52	90	10
60	90	10

Result:

The above mentioned method, Cefixime RT and all placebo RT and Blank were separated from main peak. impurities detected and separated with another impurities.

Chromatogram : 5

Trial : 5



Chromatograph conditions

Column : Novapak C18, 150 X 3.9 mm, 4 µm

Flow rate : 1.0 ml / minute

Injection volume : 10 µl

Detector Wave length : 254 nm

Column temperature : 40°C

Run time : 60 min

Gradient program

Time (min)	A (%)	B (%)
0	90	10
15	90	10
50	70	30
52	90	10
60	90	10

Preparation of Buffer:

Mix 33 mL of Tetrabutyl ammonium hydroxide solution (40% in water) in 1000 ml of water. Adjust the pH to 6.5 using orthophosphoric acid. Filter through 0.45 µ membrane filter.

Preparation of mobile phase:

Mobile Phase A : Buffer

Mobile Phase B : Acetonitrile

Preparation of Solution A:

Dissolve 6.8g of monobasic potassium phosphate in water to make 500 ml of miliq water and filter through 0.45um membrane filter

Preparation of Diluent:

Dissolve 7.1 g of anhydrous dibasic sodium phosphate in water to make 500 ml of solution. Adjust a volume of this solution with a sufficient volume of monobasic potassium phosphate (Solution A) solution to a pH of 7.0.

Preparation of system suitability solution:

Weigh accurately and transfer about 25.0 mg of Cefixime working standard into a 25 mL volumetric, dissolve and dilute to the volume with mobile phase. Heat this solution on water bath for 45 minutes (In situ preparation of Cefixime E-isomer), cool and use.

Preparation of standard stock solution:

Weigh accurately and transfer about 25.0 mg of Cefixime working standard into a 25 mL volumetric, dissolve and dilute to the volume with mobile phase.

Preparation of standard solution:

Pipette out 1 mL of standard stock solution into a 100 mL volumetric flask and dilute to volume with mobile phase (Conc.:10 ppm of cefixime).

Placebo solution:

Reconstitute the placebo with water. Weigh accurately the reconstituted placebo solution (equivalent to 100 mg of cefixime) into a 100 mL volumetric flask. Dilute to volume with diluent and mix well. Centrifuge this solution at 2500 RPM for 10 mins. Use the supernatant solution.

Sample solution:

Reconstitute the sample upto the mark with water. Weigh accurately the reconstituted solution (equivalent to 100 mg of cefixime) into a 100 mL volumetric flask. Dilute to volume with diluent and mix well. Centrifuge this solution at 2500 RPM for 10 mins. Use the supernatant solution. (Conc.: 1000 ppm of cefixime)

Procedure

Inject 10 µl of diluent as blank, System suitability solution**,** Standard solution, placebo solution and sample solution into the chromatograph, record the chromatogram and measure the peak response. The related sequence as mentioned below table.

Name of the Solution	Number of Injection
Blank (Diluent)	1
System suitability solution	1
Standard Solution	5
Placebo solution	1
Sample Solution	1
Standard Solution (Bracketing standard)	1

Note: Inject bracketing standard after every six injections of the test preparation or end of the sequence. The area difference between each bracketing standard and average area of standard preparation should be with in ±2.0%.

Evaluation of system suitability:

1. The resolution between Cefixime and Cefixime E-isomer peaks from system suitability solution should not be less than 2.0.

2. % RSD for five replicate injections of standard solution should not be more than 2.0.

Related Substance Of Proposed Method:

Procedure:

Separately inject both the standard and sample preparations into liquid chromatogram and record the peak area responses. The % RSD is not more than 2.0.

Calculation

Note: Disregard the peaks with area % less than 0.05% and the peaks due to blank and placebo.

Calculate the percentage of individual impurities in the portion of Cefixime for oral suspension taken as follows:

$$\% \text{ of individual Imp} = \frac{At \times Ws \times X1 \times 100 \times P \times d \times D \times 100}{As \times 25 \times 100 \times W \times 100 \times L}$$

% of total imp = Sum of % of all individual impurities

Where,

At = Peak response for individual impurity from the test solution

As = Average Peak response for cefixime from the standard solution

Ws = Standard weight in mg

d = Weight per ml (Density) of the oral suspension in mg

W = Weight of oral suspension taken in mg

L = Label claim in mg

P = Purity in as such basis

D = Dose, 5 ml

8. VALIDATION OF RP-HPLC METHOD

After development of HPLC method for the estimation of the Single component dosage forms validation of the method was carried out. This section describes the procedure followed for the validation of the developed method.

8.1 SOLUTION STABILITY

Performed the solution stability of standard and test preparation as per the given the method of analysis. Kept the standard preparation and test preparation on bench top analyse initially and different time intervals up to 24 hrs. Calculated the % difference of impurities in sample solution and the % RSD for standard solution response, tabulatet the results in the table given below.Its shown chromatogram no:(1-2)

Table no:1 Solution stability impurity values given below the table

Time in hours	**E- isomer**		**Highest unknown Impurity**		**Total Impurities**	
	% Impurity	**% Difference from Initial**	**% Impurity**	**% Difference from Initial**	**% Impurity**	**% Difference from Initial**
Initial	0.084	NAP	0.468	NAP	2.06	NAP
4	0.086	2.4	0.462	1.3	2.10	1.9
8	0.086	2.4	0.457	2.4	2.21	7.6
12	0.093	10.7	0.438	6.4	2.35	14.3
16	0.093	10.7	0.466	0.4	2.50	21.5
20	0.094	11.9	0.478	-2.1	2.62	27.3
24	0.085	1.2	0.437	6.6	2.60	26.5

Table no :2 Solution stability values given below

Time in hours	**Response of standard solution**
Initial	2842486
4 th Hour	2854792
8 th Hour	2791283
12 th Hour	2852277
16 th Hour	2824500
20 th Hour	2807373
24 th Hour	2827914
Mean	2828785
SD	25687.314
% RSD	0.9

Acceptance Criteria

1. The difference between initial and bench top stability sample for % of known impurity, Highest unknown impurity and Total Impurities should be ±15.0 %.

2. The% RSD of peak responses between initial and bench top stability for diluted standard not more than 2.0.

Conclusion

The % RSD of peak area of standard solution from initial to 24 Hours was found within the limits. The % difference of % of impurity for sample solution is failing at 16 th Hour. From the above study, it was established that the Standard solution is stable for a period of about 24 Hours and sample solution is stable for a period of about 12 Hours at bench top.

8.2 SYSTEM SUITABILITY STUDIES

System suitability studies were carried out as specified in the United States Pharmacopoeia (USP). These parameters include column efficiency, resolution, tailing factor and RSD were calculated in present study.

Prepared Standard Preparations as per test procedure and made six replicate injections. Evaluated system suitability parameters as per the test procedure and tabulated the results in the table given below. Its shown chromatogram no:(3)

Table no:3 System suitability parameters

System Suitability Parameters	Observed value	Acceptance criteria
Resolution between Cefixime and Cefixime (E)-isomer obtained from system suitability solution	3.0	NLT 2.0
The relative standard deviation obtained from six replicate injections of standard solution	0.7	NMT 2.0

Table no: 4 Response of the Standard replicate injections

No of injection	Response
	Cefixime
01	355423
02	351334
03	350899
04	352759
05	352560
06	357365
Mean	353390
Stdev.	2508.107
% RSD	0.7

Acceptance criteria:

1. The resolution between Cefixime and Cefixime E-isomer peaks from system suitability solution is not less than 2.0.
2. % RSD for six replicate injections of standard solution should not be more than 2.0.

Conclusion: The System suitability parameters are within the limit.

8.3 SPECIFICITY

The following methods were employed for demonstrating specificity for HPLC method. In the first method, the conditions of HPLC method developed, namely, percentage of the organic solvent in mobile phase, pH of the mobile phase, flow rate, etc. were changed in HPLC and the presence of additional peaks, if any, was observed. The second method involves the peak purity test method using diode array detector. The diode array derivative spectrums and derivative chromatograms of the standard and sample drug peaks were recorded and compared. The third method was based on measurement of the absorbance ratio of the drug peaks at different wavelengths Its shown chromatogram no:(4-8).

Placebo interference

Specificity is the ability of the method to measure the analyte in the presence of matrix components. The Specificity will be demonstrated by injecting the solutions of blank, placebo mixture, standard and sample solution. The interference with placebo mixture is checked.

Table no: 5 Summarized the results in the Cefixime table given below.

Sample ID	**Interference (Cefixime)**	
	RT (min)	**Peak purity**
Blank	Nil	NA
Placebo	Nil	NA
Standard with placebo solution	32.22	1.0
Standard solution	32.83	1.0
Test Sample	32.49	1.0

Table no: 6 Summarized the results in the Cefixime E-isomer table given below

Sample ID	Interference (Cefixime E-isomer)	
	RT (min)	**Peak purity**
Blank	Nil	NA
Placebo	Nil	NA
Standard with placebo solution	30.27	1.0
Test Sample	30.53	1.0

Acceptance Criteria

1. There should not be any interference of blank, placebo peaks at the Retention Time (RT) of main peak and known impurity peaks.
2. The Peak Purity should be not less than 0.9 in open lab software / purity angle should less than purity threshold for Empower Software.

Conclusion

The above observation reveals that no interference of any of the blank and placebo was observed at the retention time (RT) of main peak and known impurity peaks.

8.4 LIMIT OF DETECTION (LOD)

Limit of detection is the lowest concentration of the analyte that can be detected by injecting decreasing amount, not necessarily quantity by the method, under the stated experimental conditions. The minimum concentration at which the analyte can be detected is determined from the linearity curve by applying the formula.

$$\text{Limit of detection} = \frac{\sigma}{S} \times 3.3$$

LIMIT OF QUANTITATION (LOQ)

Limit of Quantitation is the lowest concentration of the analyte in a sample that can be estimated quantitatively. By injecting decreasing amount of drug, with acceptable precision and accuracy under the experimental conditions of the method.

Limit of Quantitation can be obtained from linearity curve by applying the following formula.

$$\text{Limit of Quantitation} = \frac{\sigma}{S} X10$$

Limit Of Detection and Limit Of Quantitation

Prepared and injected different concentration of Cefixime (0.01 % to 0.06% of working concentration) from standard stock solution and determined the LOD, LOQ by residual standard deviation method. Results are summarized in the below table. Its shown chromatogram no:(9-10)

Table no: 7 LOD and LOQ values given below

Sr. no.	Level (%)	Concentration in µg/ml (ppm) Cefixime	Response of Cefixime peak
01	0.01	0.1	6619
02	0.02	0.2	9462
03	0.03	0.3	13959
04	0.04	0.4	15808
05	0.05	0.5	17448
06	0.06	0.6	19303
σ (standard deviation)			1035.787
Slope			25493.43

Table no: 8 Result of LOD and LOQ values given below

LOD		LOQ	
% with respect to test concentration	ppm	% with respect to test concentration	ppm
0.01	0.1	0.04	0.4

Precision at LOD and LOQ:

Injected LOD and LOQ solution (6 replicates), Calculated percentage RSD. The results are summarized in the table given below.

Table no: 9 Precision of LOD and LOQ values given below

No of Injection	LOD	LOQ
01	8034	15432
02	8515	15069
03	5952	15078
04	6811	15889
05	6999	15357
06	4904	15347
Mean	6869	15362
Stdev.	1325.814	299.795
% RSD	19.3	2.0

Acceptance criteria

1. The % Relative standard deviation for six replicate LOD level areas should be NMT 30.0%.
2. The % Relative standard deviation for six replicate LOQ level areas should be NMT 10.0%.

Conclusion:

The Relative Standard Deviation for Limit of Detection and Relative Standard Deviation results for limit of quantification were found within limits.

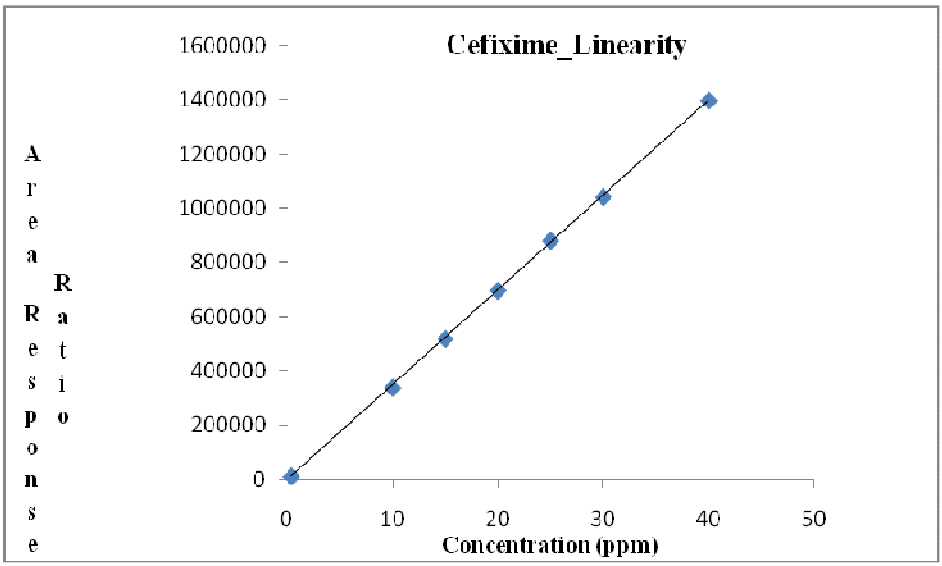
8.5 LINEARITY & RANGE

Ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Performed the linearity in the concentrations at LOQ, 50%, 75%, 100%, 125%, 150% , 175% and 200% of specification limit. Recorded the area for each level and calculate slope, y- intercept & coefficient of correlation and coefficient of regression. Plotted the graph of Cefixime concentration on X- axis and area response on Y-axis. Summarized the results in the below table. Its shown chromatogram no:(11-14)

Table no: 10 Linearity of the sample calculation given below

Sr. No.	% Level	Concentration in µg/ml (ppm)	Peak Response of Cefixime
01	LOQ	0.4	10371
02	50	10	338460
03	75	15	518872
04	100	20	697549
05	125	25	881632
06	150	30	1043549
07	200	40	1399437
Slope			**35170.1295**
Y intercept			**-6859.4550**
Coefficient of correlation			**0.9999**
Coefficient of regression			**0.9999**
Y intercept should be ± 5.0% of the active response at 100% concentration			**1.0**

Fig no : 2 Linearity of Cefxime



(fig : 2)

Acceptance Criteria:

1. The Coefficient of correlation should not be less than 0.995.
2. The Y intercept should be ± 5.0% of the active response at 100% concentration.

Conclusion

The detector response was found linear with a Coefficient of correlation of 0.9999 and Coefficient of regression 0.9999 for Cefixime shows that the related substances method was meeting the linearity and range acceptance criteria.

8.6 ACCURACY

Accuracy of the method was determined by recovery experiments. To the formulation, the reference standards of the respective drugs were added at the level of 100 %. These were further diluted by procedure as followed in estimation of formulation. The resulting sample solutions were analyzed by HPLC. The amount of the each drug present, percentage recovery, percentage relative standard deviation (% RSD) was calculated. The percentage recovery was calculated using the formula,

$$\text{Percentage recovery} = \frac{[a+b]-a}{b} x\,100$$

Accuracy is the closeness of the test results obtained by the method to the true value. Accuracy may often be expressed as percent recovery by the assay of known, added amounts of analyte. Accuracy is a measure of the exactness of analytical method.

Injected triplicate preparations by spiking Cefixime on placebo from LOQ, 50%, 100%, 150% and 200% with respect to target concentration. Calculated the % Recovery for Cefixime. Summarized the results in the table given below. Its shown chromatogram no:(15-18)

Table no: 11 Accuracy of the product in the table given below

Series	No of Sample	Added in ppm	Found in ppm	Recovery in %	Average in %
LOQ	**01**	0.401	0.398	99.3	98.1
	02	0.401	0.388	96.8	
	03	0.401	0.393	98.1	
50%	**01**	10.020	10.075	100.5	**100.9**
	02	10.020	10.169	101.5	
	03	10.020	10.105	100.8	
100%	**01**	20.040	20.029	99.9	**98.8**
	02	20.040	20.042	100.0	
	03	20.040	19.359	96.6	
150%	**01**	30.060	29.288	97.4	**98.9**
	02	30.060	30.206	100.5	
	03	30.060	29.689	98.8	
200%	**01**	40.080	37.962	94.7	**95.0**
	02	40.080	36.644	91.4	
	03	40.080	39.598	98.8	
			Mean	98.5	
			Stdev.	2.431	

	% RSD	2.5	

Acceptance Criteria

1. The % Recovery at 50 % to 200% level should not be less than 90.0% and not more than 110.0%.
2. The % Recovery at LOQ level should not be less than 80.0% and not more than 120.0%.

Conclusion:

The % Recovery for Cefixime (unknown impurity) were found within the limits. Comparison of above results meeting the accuracy acceptance criteria.

8.7 PRECISION

- **Method Precision (Repeatability)**

To demonstrate the method precision of the related substances method by analyzing six replicates of sample preparation. Calculated the mean value, the standard deviation and the relative standard deviation for known impurity, Highest unknown impurity and Total Impurities. Summarized the results in the table given below. Its shown chromatogram no:(19-20)

Table no: 12 Repeatability of the product results in the table given below

No. of Sample	**Method Precision**				
	E- Isomer		**Highest unknown impurity**		**Total Impurities**
	RRT	**%**	**RRT**	**%**	**%**
01	0.94	0.10	1.07	0.31	1.84
02	0.94	0.10	1.07	0.30	1.81
03	0.94	0.10	1.07	0.29	1.51
04	0.94	0.09	1.07	0.29	1.73
05	0.94	0.08	1.07	0.29	1.66
06	0.94	0.10	1.07	0.28	1.74

Mean		0.09		0.29	1.72
SD		0.006		0.011	0.119
% RSD		6.5		3.7	6.9

Acceptance Criteria

The % RSD of known impurity, Highest unknown impurity and Total Impurities should not be more than 10.0.

Conclusion

The % RSD of % of known impurity, highest unknown impurity and total impurities obtained from six preparations of sample solution were found within the limits.

- **Intermediate precision**

Performed the procedure as detailed in the method precision study on a different day, by a different analyst, preferably using a different instrument and with freshly prepared mobile phase, sample and standard preparation. Prepared the test solution in replicate (six Preparations) using the same batch, which is taken for method precision, study.

Calculated the mean value, the standard deviation, the relative standard deviation for known impurity, Highest unknown impurity and Total Impurities. Summarized the results in the table given below Its shown chromatogram no:(21-22)

Table no: 13 Intermediate precision of the product results in the table given below

No. of Sample	**Intermediate Precision**				
	E- Isomer		**Highest unknown impurity**		**Total Impurities**
	RRT	**%**	**RRT**	**%**	**%**
01	0.94	0.09	1.07	0.31	1.52
02	0.94	0.09	1.07	0.32	1.44
03	0.94	0.09	1.07	0.31	1.41
04	0.94	0.09	1.06	0.32	1.52

05	0.96	0.09	1.06	0.30	1.51
06	0.94	0.08	1.06	0.31	1.52
Mean		0.09		0.31	1.49
SD		0.003		0.006	0.048
% RSD		4.0		1.9	3.2

Table no: 14 Comparison of method precision and intermediate precision results:

No. of Sample		**Overall % RSD**				
		E- Isomer		**Highest unknown Impurity**		**Total Impurities**
		RRT	**%**	**RRT**	**%**	**%**
Method precision	**01**	0.94	0.10	1.07	0.31	1.84
	02	0.94	0.10	1.07	0.30	1.81
	03	0.94	0.10	1.07	0.29	1.51
	04	0.94	0.09	1.07	0.29	1.73
	05	0.94	0.08	1.07	0.29	1.66
	06	0.94	0.10	1.07	0.28	1.74
Intermediate precision	**07**	0.94	0.09	1.07	0.31	1.52
	08	0.94	0.09	1.07	0.32	1.44
	09	0.94	0.09	1.07	0.31	1.41
	10	0.94	0.09	1.06	0.32	1.52
	11	0.96	0.09	1.06	0.30	1.51
	12	0.94	0.08	1.06	0.31	1.52
Overall Mean (n=12)			0.09		0.30	1.60
SD			0.006		0.013	0.147

% RSD		6.8		4.3	9.2

Acceptance Criteria

1. The % RSD of known impurity, Highest unknown impurity and Total Impurities should not be more than 10.0.
2. The over all % RSD of known impurity, Highest unknown impurity and Total Impurities obtained from method precision and intermediate precision results should not be more than 10.0.

Conclusion:

The % RSD of % of known impurity, Highest unknown impurity and Total Impurities obtained from six preparations of sample solution were found within the limits. Comparison of the results obtained by two different days with different analysts and different instruments, shows that the related substances method was meeting the Intermediate precision acceptance criteria.

8.8 ROBUSTNESS

The robustness of an analytical method 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.

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied. The separation factor, retention times and peak symmetry were then calculated.

- **Effect of variation in column oven temperature**

To demonstrate the robustness of test method, prepared and injected standard solution and sample solution at 35°C and at 45°C of column oven temperature. Calculated the overall % RSD for known impurity, highest unknown impurity and total impurity obtained from method precision and robustness results. Summarized the results in the below table. Its shown chromatogram no:(23-24)

Table no: 15 Robustness system suitability values of Colum temperature variation

System Suitability Parameters	**Observed value with Column temperature**			**Acceptance criteria**
	35°C	**40°C**	**45°C**	

Resolution between Cefixime and Cefixime (E)-isomer obtained from system suitability solution	4.3	3.0	3.9	NLT 2.0
The relative standard deviation obtained from five replicate injection standard solution	1.5	0.7	1.3	NMT 2.0

Table no: 16 Robustness values of Low column oven temperature (35°C)

No. of Sample		**Overall % RSD**				
		E-isomer		**Highest unknown Impurity**		**Total treImpurities**
		RRT	**%**	**RRT**	**%**	**%**
Method precision	**01**	0.94	0.10	1.07	0.31	1.84
	02	0.94	0.10	1.07	0.30	1.81
	03	0.94	0.10	1.07	0.29	1.51
	04	0.94	0.09	1.07	0.29	1.73
	05	0.94	0.08	1.07	0.29	1.66
	06	0.94	0.10	1.07	0.28	1.74
Robustness	**07**	0.9	0.079	1.1	0.338	1.60
	08	0.9	0.08	1.1	0.306	1.62
Overall Mean (n=8)			0.09		0.30	1.69
SD			0.01		0.02	0.11
% RSD			9.20		6.34	6.67

Table no: 17 Robustness values of High column oven temperature (45°C)

No. of Sample		Overall % RSD				
		E-isomer		Highest unknown Impurity		Total Impurities
		RRT	%	RRT	%	%
Method precision	01	0.94	0.10	1.07	0.31	1.84
	02	0.94	0.10	1.07	0.30	1.81
	03	0.94	0.10	1.07	0.29	1.51
	04	0.94	0.09	1.07	0.29	1.73
	05	0.94	0.08	1.07	0.29	1.66
	06	0.94	0.10	1.07	0.28	1.74
Robustness	07	0.9	0.11	1.1	0.27	1.63
	08	0.9	0.09	1.1	0.26	1.61
Overall Mean (n=8)			0.10		0.28	1.69
SD			0.01		0.02	0.11
% RSD			8.51		5.65	6.49

Acceptance criteria:

The over all % RSD of Known impurity, Highest unknown Impurity and Total Impurities obtained from method precision and robustness results should not be more than 10.0.

Conclusion:

The overall % RSD of % of known impurity, Highest unknown impurity and Total Impurities obtained from method precision and robustness results meeting the acceptance criteria. The above study indicates that column oven temperature from 35°C to 45°C is suitable.

- **Effect of variation in flow rate**

To demonstrate the robustness of test method, prepared and injected standard solution and sample solution at 0.8 mL/min and at 1.2 mL/min of flow rate. Calculated the overall % RSD for known impurity, highest unknown impurity and total impurity obtained from method precision and robustness results. Summarized the results in the below table Its shown chromatogram no:(25-26)

Table no: 18 Robustness system suitability values of flow rate variation

System Suitability Parameters	Observed value with Flow rate			Acceptance criteria
	0.8 mL /min	1.0 mL/min	1.2 mL/min	
Resolution between Cefixime and Cefixime (E)-isomer obtained from system suitability solution	3.0	3.0	3.2	NLT 2.0
The relative standard deviation obtained from five replicate injections of standard solution	0.5	0.7	0.6	NMT 2.0

Table no: 19 Robustness values of Low flow rate(0.8ml/min)

No. of Sample		Overall % RSD				
		E-isomer		Highest unknown Impurity		Total Impurities
		RRT	%	RRT	%	%
Method precision	01	0.94	0.10	1.07	0.31	1.84
	02	0.94	0.10	1.07	0.30	1.81
	03	0.94	0.10	1.07	0.29	1.51
	04	0.94	0.09	1.07	0.29	1.73

	05	0.94	0.08	1.07	0.29	1.66
	06	0.94	0.10	1.07	0.28	1.74
Robustness	**07**	0.94	0.085	1.07	0.313	1.81
	08	0.94	0.087	1.07	0.337	1.81
Overall Mean (n=8)			0.09		0.30	1.74
SD			0.01		0.02	0.11
% RSD			6.80		6.41	6.31

Table no: 20 Robustness values of High flow rate(1.2ml/min)

No. of Sample		**Overall % RSD**				
		E-isomer		**Highest unknown Impurity**		**Total Impurities**
		RRT	**%**	**RRT**	**%**	**%**
Method precision	**01**	0.94	0.10	1.07	0.31	1.84
	02	0.94	0.10	1.07	0.30	1.81
	03	0.94	0.10	1.07	0.29	1.51
	04	0.94	0.09	1.07	0.29	1.73
	05	0.94	0.08	1.07	0.29	1.66
	06	0.94	0.10	1.07	0.28	1.74
Robustness	**07**	0.93	0.089	1.07	0.246	1.89
	08	0.93	0.088	1.07	0.248	1.99
Overall Mean (n=8)			0.09		0.28	1.77
SD			0.01		0.02	0.15
% RSD			6.13		8.03	8.31

Acceptance criteria:

The over all % RSD of known impurity, highest unknown impurity and total impurity obtained from method precision and robustness results should not be more than 10.0.

Conclusion:

The overall % RSD of % of known impurity, Highest unknown impurity and Total Impurities obtained from method precision and robustness results meeting the acceptance criteria. The above study indicates that flow rate from 0.8mL/min to 1.2 mL/min is suitable.

- **Effect of variation in mobile phase pH**

To demonstrate the robustness of test method, prepared and injected standard solution and sample solution at pH of 6.3 and 6.7 of mobile phase pH. Calculated the overall % RSD for known impurity, highest unknown impurity and total impurity obtained from method precision and robustness results. Summarized the results in the below table. Its shown chromatogram no:(27-28)

Table no: 21 Robustness system suitability values mobile phase pH variation

System Suitability Parameters	**Observed value with pH**			**Acceptance criteria**
	6.3	**6.5**	**6.7**	
Resolution between Cefixime and Cefixime (E)-isomer obtained from system suitability solution	4.12	3.0	4.3	NLT 2.0
The relative standard deviation obtained from five replicate injections of standard solution	0.74	0.7	0.87	NMT 2.0

Table no: 22 Robustness mobile phase Low pH (6.3) values

No. of Sample	**Overall % RSD**				
	E-isomer		**Highest unknown Impurity**		**Total Impurities**
	RRT	**%**	**RRT**	**%**	**%**

Method precision	**01**	0.94	0.10	1.07	0.31	1.84
	02	0.94	0.10	1.07	0.30	1.81
	03	0.94	0.10	1.07	0.29	1.51
	04	0.94	0.09	1.07	0.29	1.73
	05	0.94	0.08	1.07	0.29	1.66
	06	0.94	0.10	1.07	0.28	1.74
Robustness	**07**	0.94	0.318	1.07	0.318	1.76
	08	0.94	0.333	1.07	0.333	1.77
Overall Mean (n=8)			0.10		0.30	1.73
SD			0.01		0.02	0.10
% RSD			7.65		6.25	5.99

Table no: 23 Robustness mobile phase High pH (6.7) values

No. of Sample		**Overall % RSD**				
		E-isomer		**Highest unknown Impurity**		**Total Impurities**
		RRT	**%**	**RRT**	**%**	**%**
Method precision	**01**	0.94	0.10	1.07	0.31	1.84
	02	0.94	0.10	1.07	0.30	1.81
	03	0.94	0.10	1.07	0.29	1.51
	04	0.94	0.09	1.07	0.29	1.73
	05	0.94	0.08	1.07	0.29	1.66
	06	0.94	0.10	1.07	0.28	1.74
Robustness	**07**	0.94	0.089	1.07	0.257	1.72
	08	0.93	0.086	1.08	0.26	1.65

Overall Mean (n=8)		0.10		0.30	1.73
SD		0.01		0.02	0.10
% RSD		7.65		6.25	5.99

Acceptance criteria: The overall % RSD of known impurity, highest unknown impurity and total impurity obtained from method precision and robustness results should not be more than 10.0.

Conclusion:

The overall % RSD of % of known impurity, Highest unknown impurity and Total Impurities obtained from method precision and robustness results meeting the acceptance criteria.

The above study indicates that mobile phase pH 6.3 to 6.7 is suitable.

8.9 INTERFERENCE FROM DEGRADATION PRODUCTS

A study was conducted to demonstrate the effective separation of degradants from Cefixime for oral suspension USP 100 mg/5mL of related substances method. Drug product, Placebo and Blank were exposed to the following stress conditions to induce degradation Its shown chromatogram no:(29-32)

Table no:24 Degradation of the product in the table given below

Stress Condition	**Cefixime**		
	RT (min)	**% degradation**	**Peak purity**
Kept in water bath at 60°c with 5 mL of 5M HCl for 60 minutes (Acid Hydrolysis).	32.65	19.41	1.00
Kept in bench top with 5 mL of 0.5M NaOH for 5minutes (Base Hydrolysis).	31.93	12.64	1.00
Kept in bench top with 5 mL of 3% Hydrogen peroxide solution for 5 minutes (Oxidation).	31.92	5.86	1.00

Reconstituted sample was kept in room temperature for 24 hours (Water Hydrolysis).	32.39	0.49	1.00
Exposed to Dry heat at 50° C for about 6 days.	32.28	0.04	1.00
Exposed to humidity at 25°C and 90% RH for about 7 days.	32.29	0.07	1.00

Acceptance Criteria:

1. There should not be any interference of degradants at the Retention Time (RT) of main peak and known impurity peaks.
2. The Peak Purity should be not less than 0.9 in open lab software / purity angle should less than purity threshold for Empower Software.

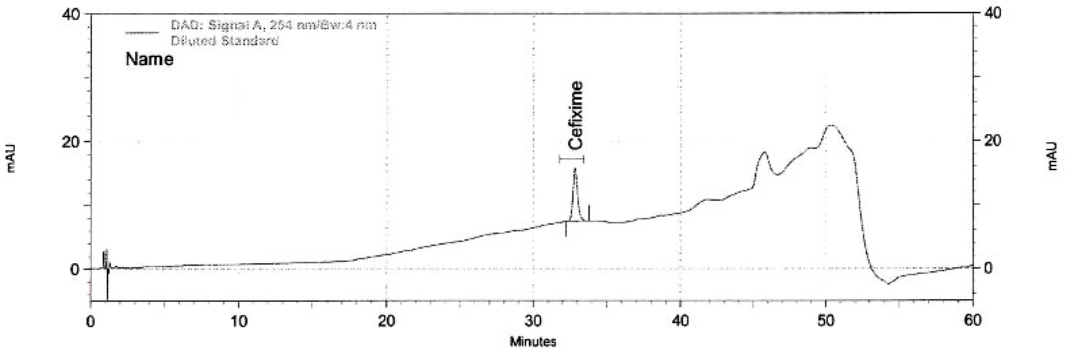
Conclusion :

The above observation reveals that no interference of degradants was observed on the area of Cefixime and all impurities as well. This demonstrates that the method is specific for Related Substances of Cefixime for oral suspension USP 100 mg/5mL.

9. VALIDATION CHROMATOGRAM

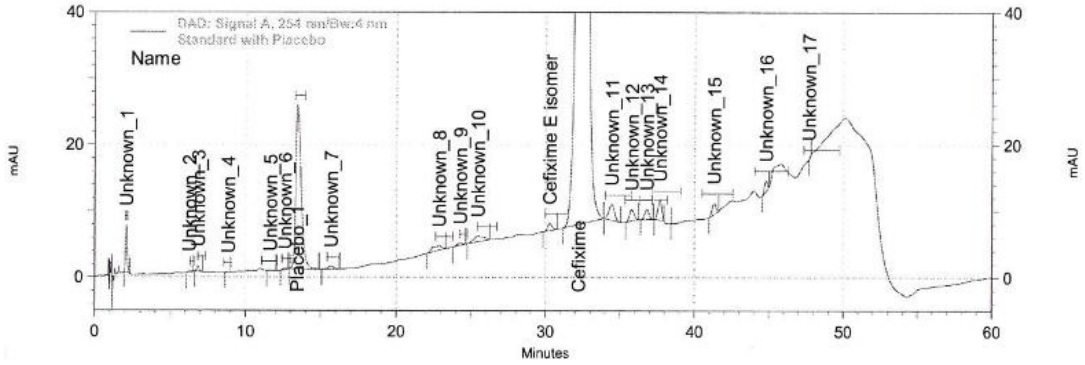
Chromatogram no: 1

A Representative chromatogram of solution stability of Dilutes Standard



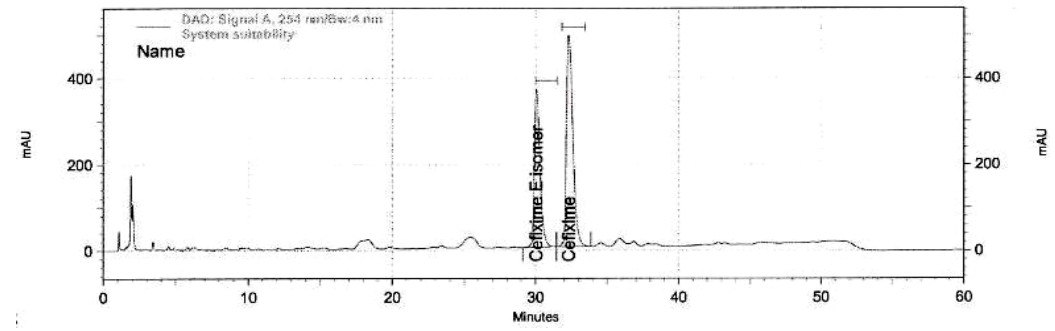
Chromatogram no: 2

A Representative chromatogram of solution stability Sample



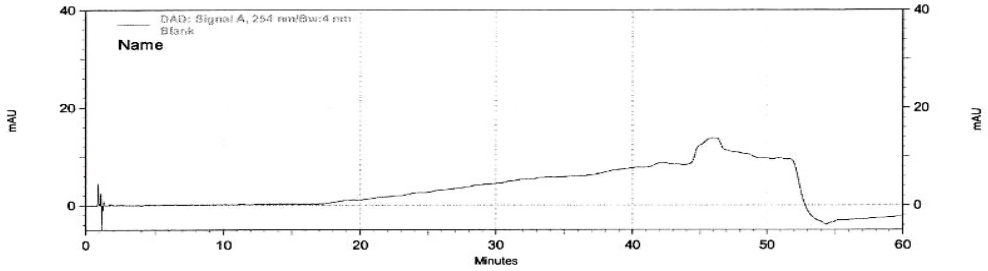
Chromatogram no: 3

Representative chromatogram of system suitability solution



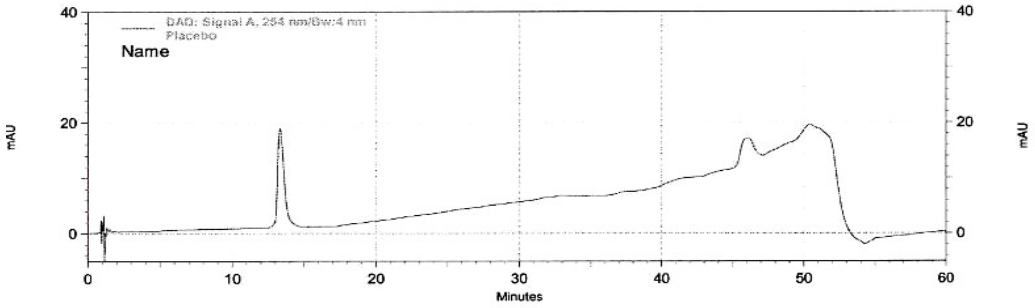
Chromatogram no: 4

A Representative Chromatogram of Specificity Blank



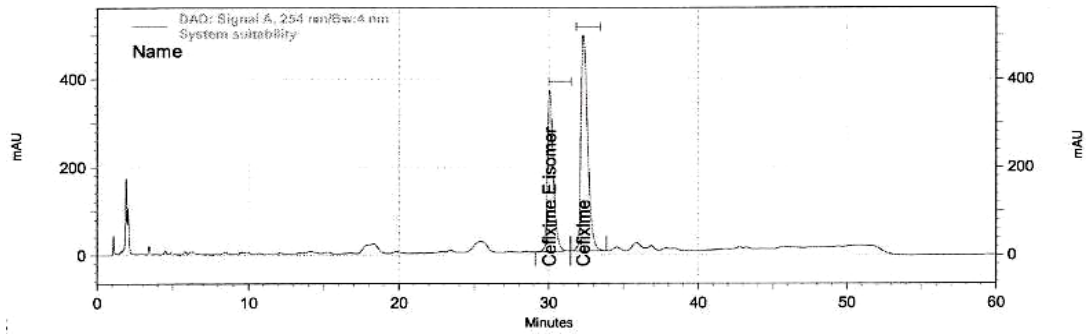
Chromatogram no: 5

A Representative Chromatogram of Specificity Placebo



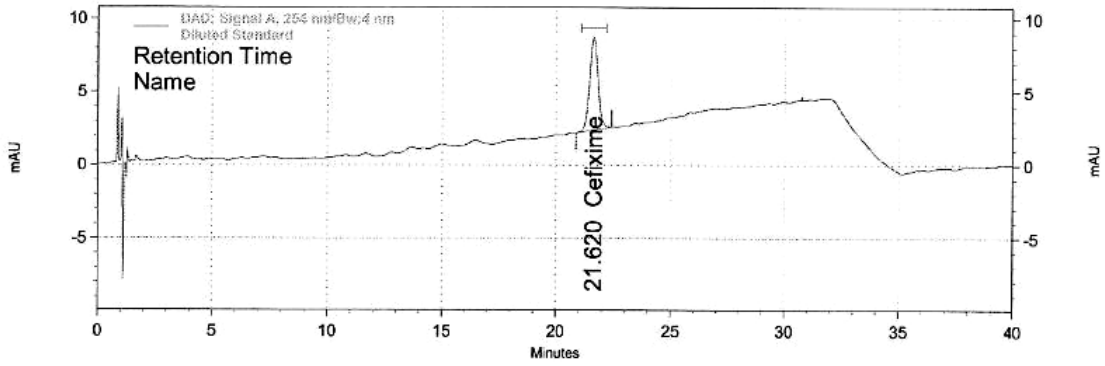
Chromatogram no: 6

A Representative Chromatogram of Specificity System suitability



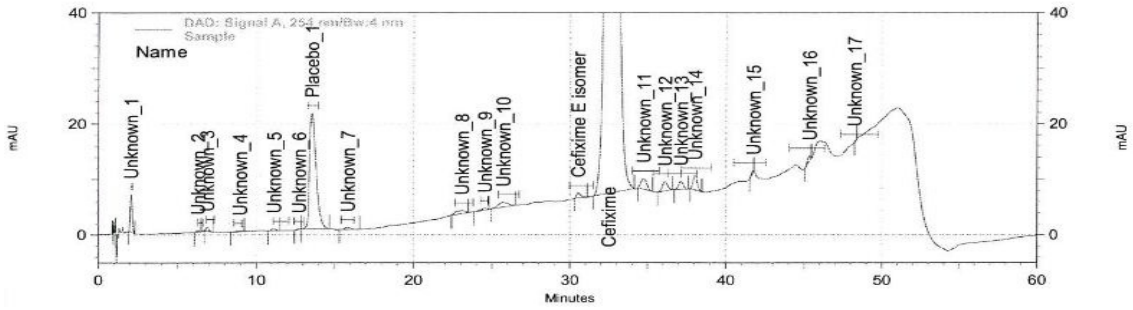
Chromatogram no: 7

A Representative Chromatogram of Specificity Diluted standard



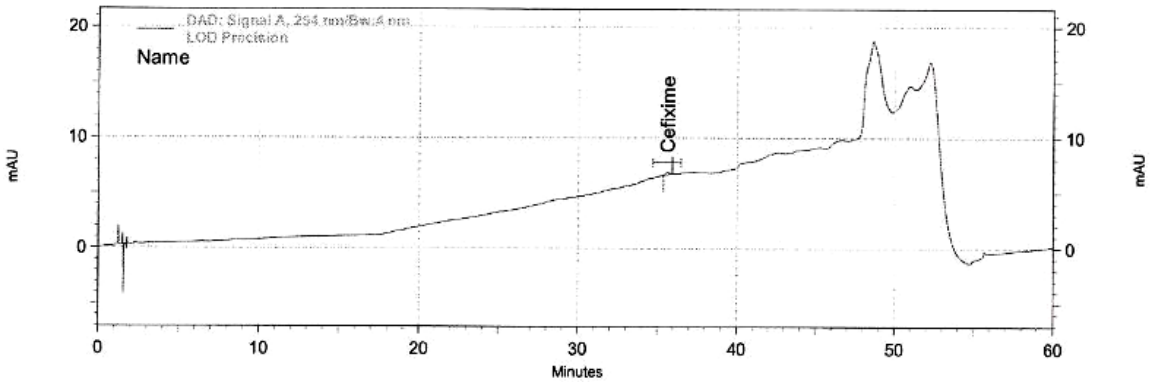
Chromatogram no: 8

A Representative Chromatogram of Specificity Sample



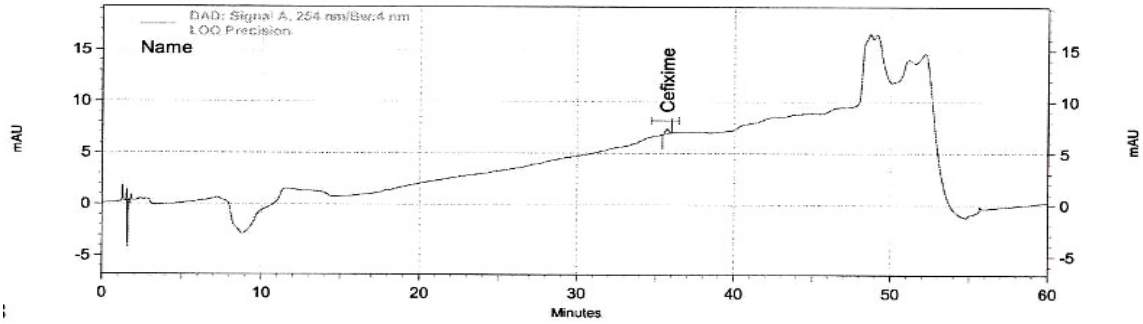
Chromatogram no: 9

A Representative chromatogram of LOD concentration of Cefixime



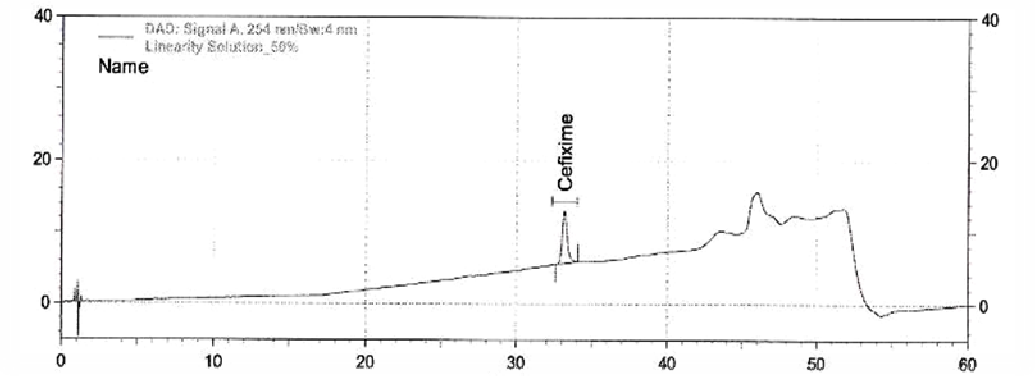
Chromatogram no: 10

A Representative chromatogram of LOQ concentration of Cefixime



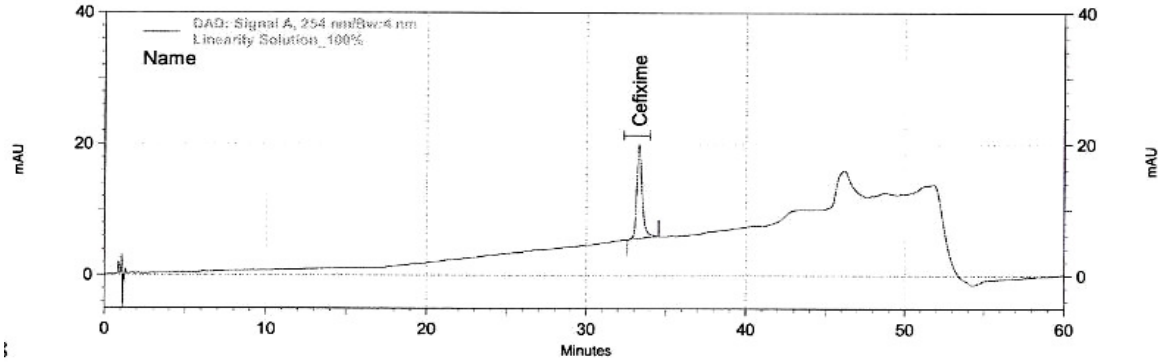
Chromatogram no: 11

A Representative chromatogram of the linearity 50% solution



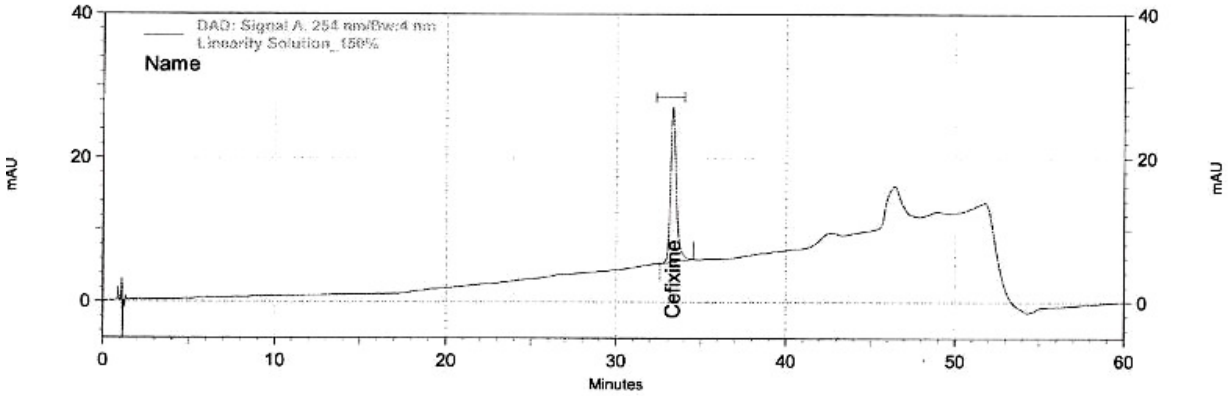
Chromatogram no: 12

A Representative chromatogram of the linearity 100% solution



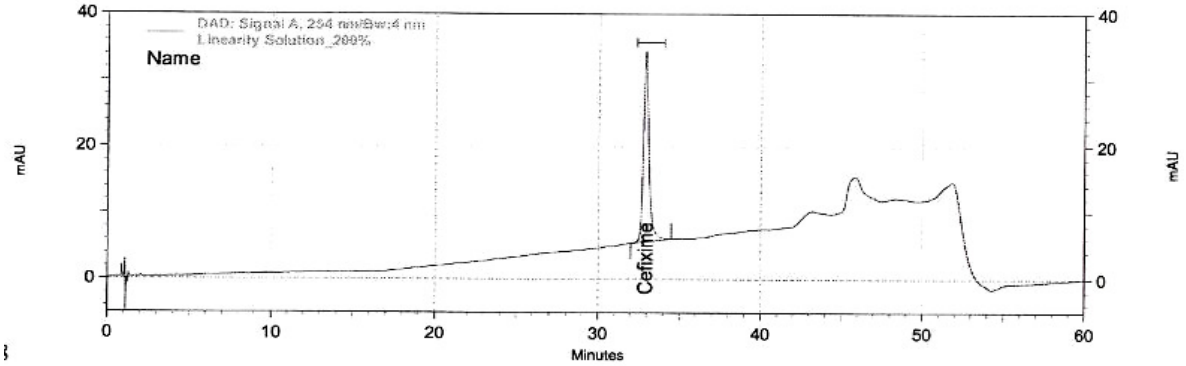
Chromatogram no: 13

A Representative chromatogram of the linearity 150% solution



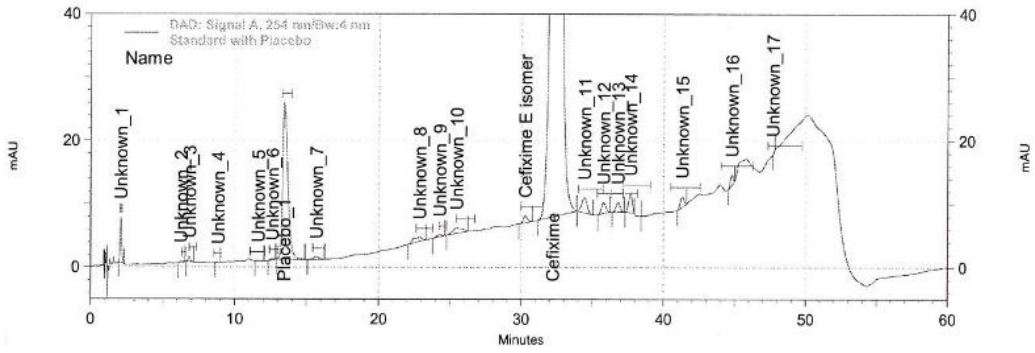
Chromatogram no: 14

A Representative chromatogram of the linearity 200% solution



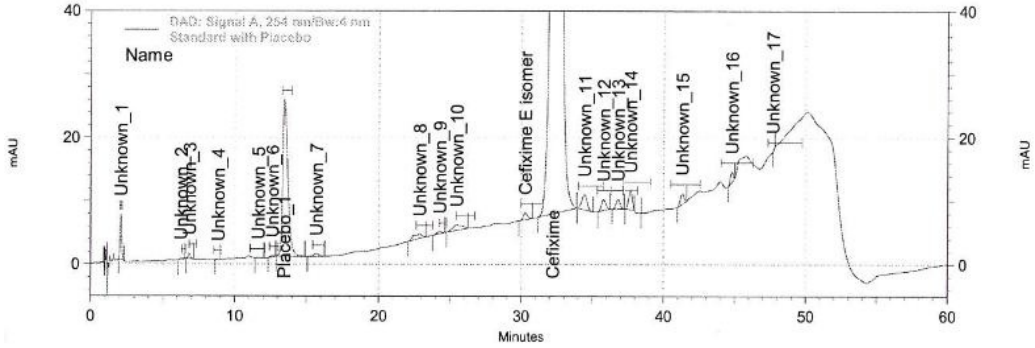
Chromatogram no: 15

A Representative chromatogram of Accuracy sample 50 %



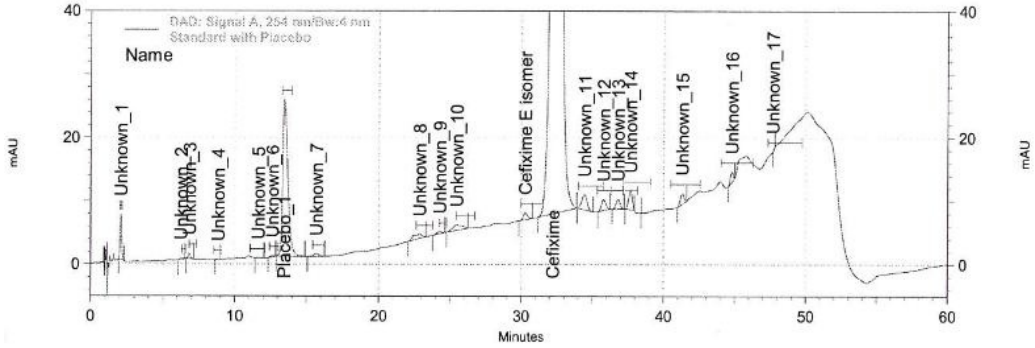
Chromatogram no: 16

A Representative chromatogram of Accuracy sample 100 %



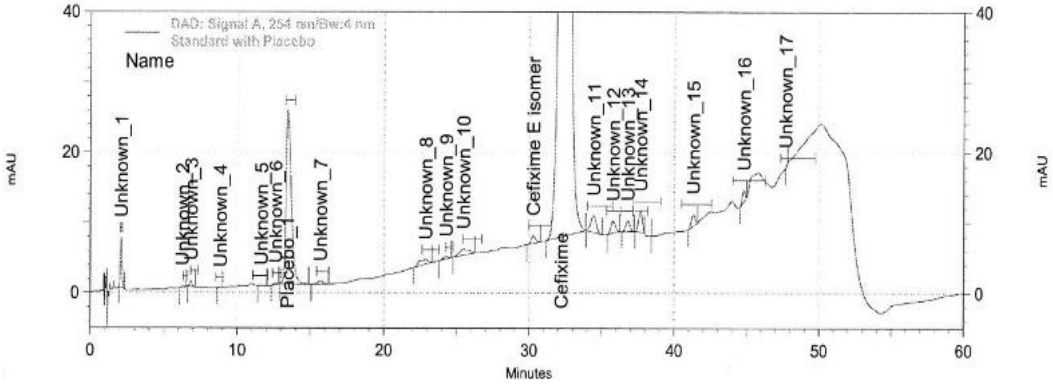
Chromatogram no: 17

A Representative chromatogram of Accuracy sample 150 %



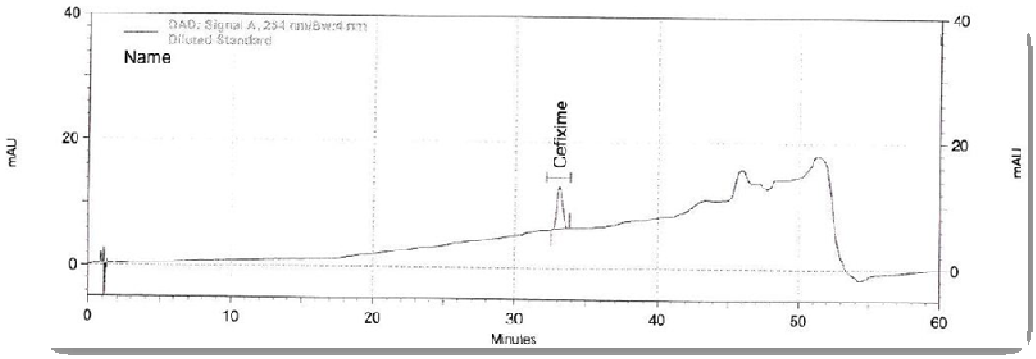
Chromatogram no: 18

A Representative chromatogram of Accuracy sample 200 %



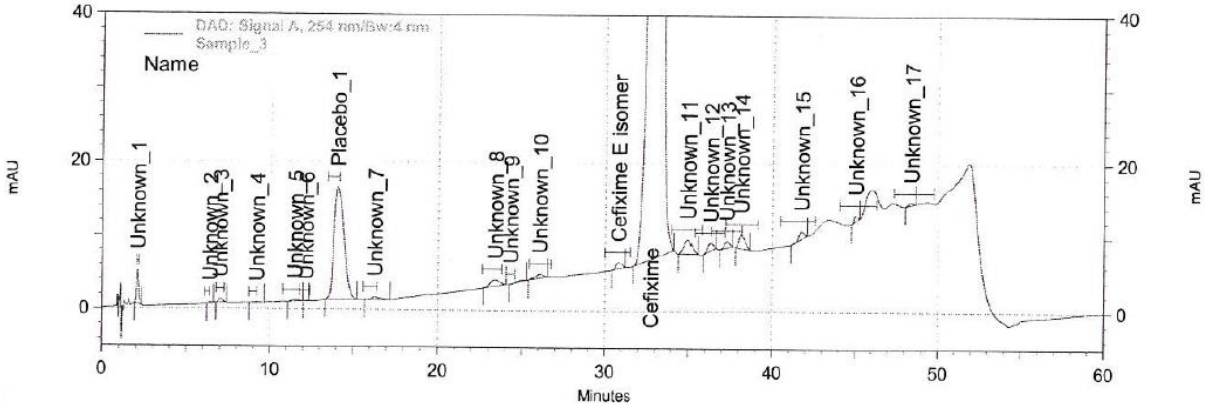
Chromatogram no: 19

A Representative chromatogram of Precision Diluted standard



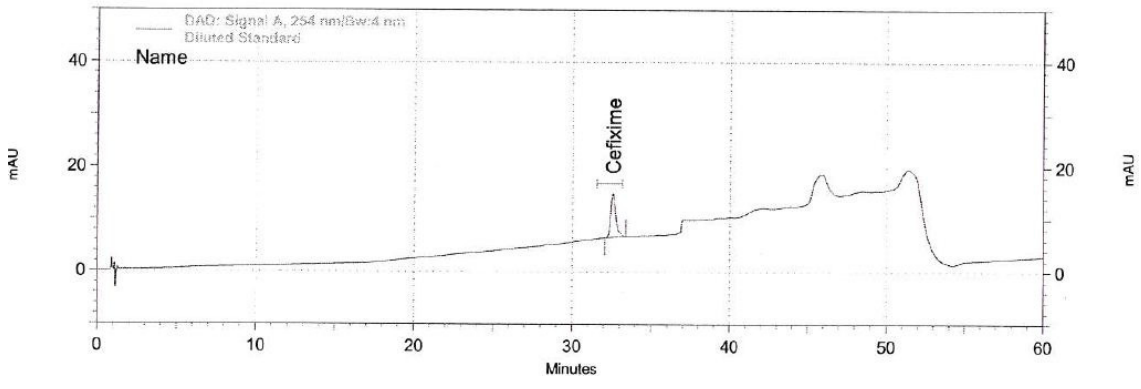
Chromatogram no: 20

A Representative chromatogram of Precision sample



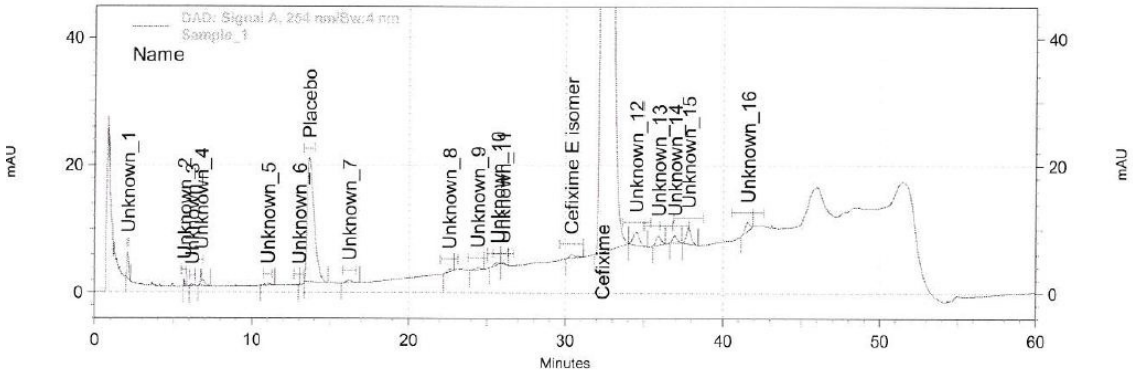
Chromatogram no: 21

A Representative chromatogram of Intermediate Precision Diluted standard



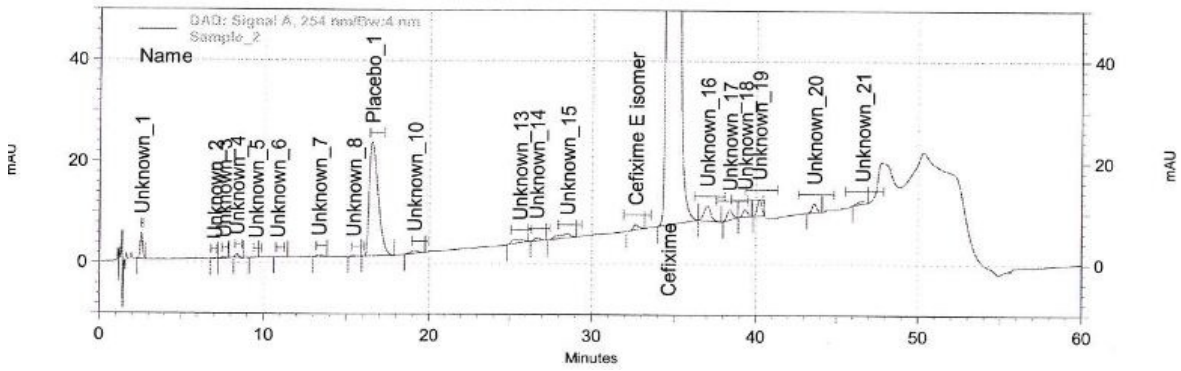
Chromatogram no: 22

A Representative chromatogram Intermediate Precision sample



Chromatogram no: 23

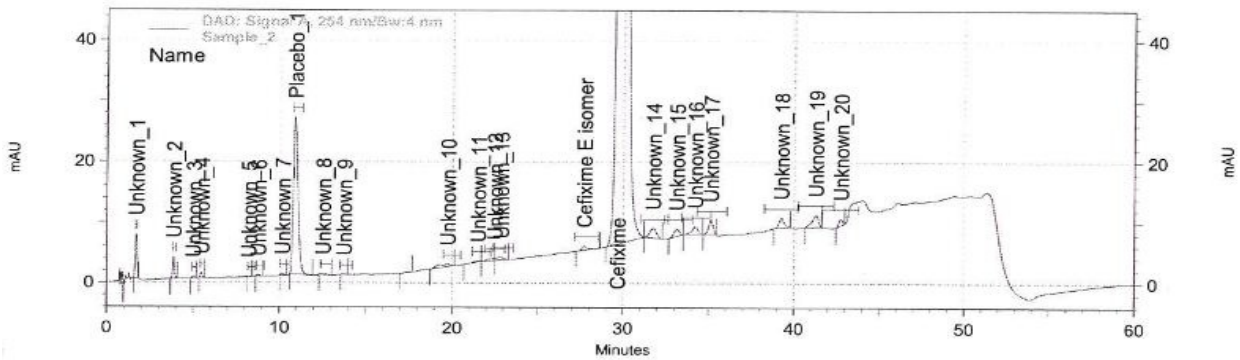
A Representative chromatogram of Robustness low column temperature



sample

Chromatogram no: 24

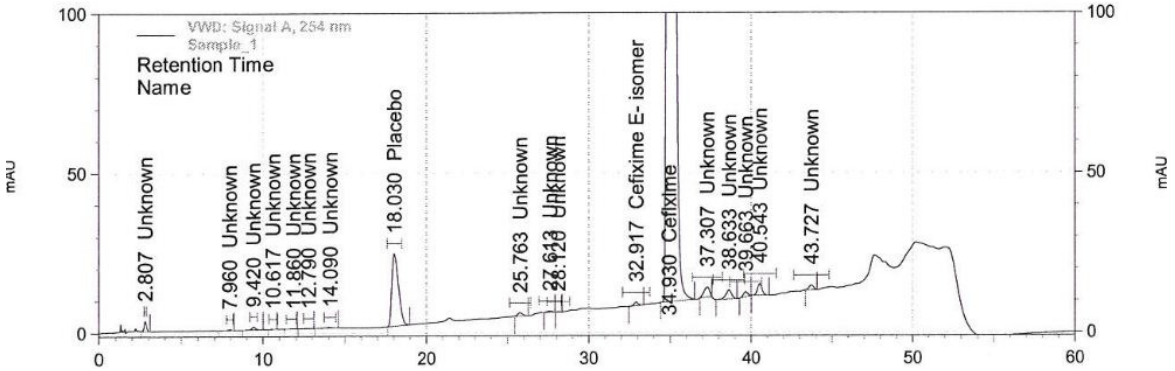
A Representative chromatogram of Robustness High column temperature



sample

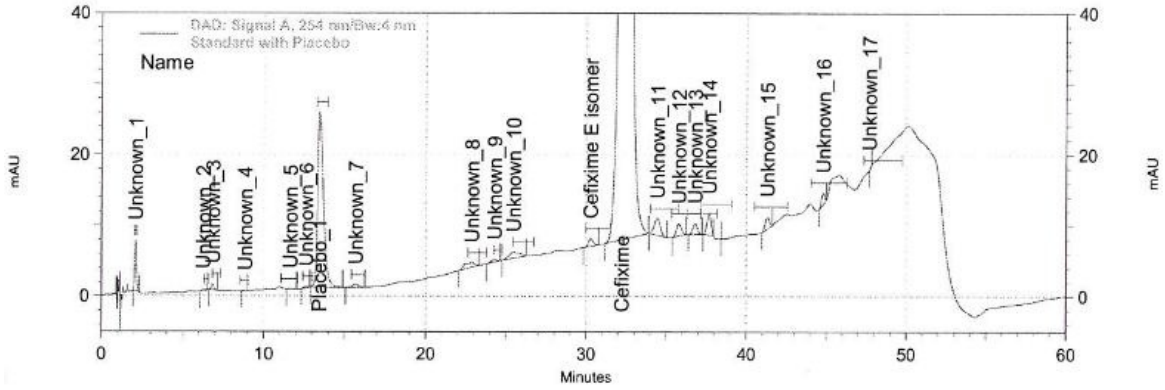
Chromatogram no: 25

A Representative chromatogram of Robustness low flow rate sample



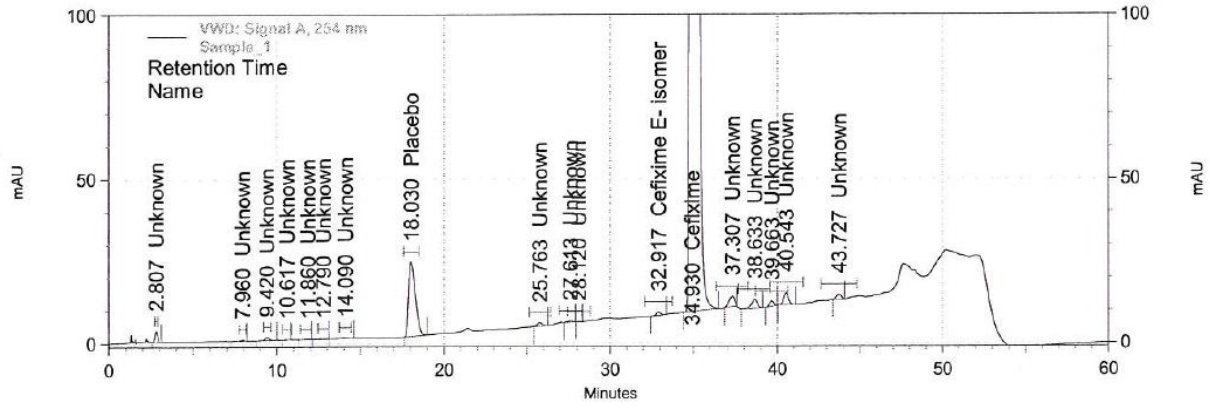
Chromatogram no: 26

A Representative chromatogram of Robustness High flow rate sample



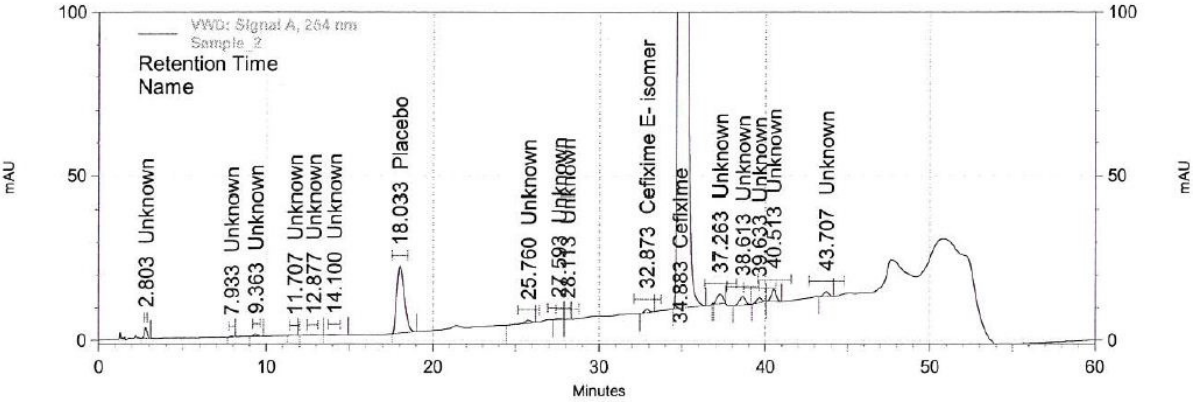
Chromatogram no: 27

A Representative chromatogram of Robustness low pH sample



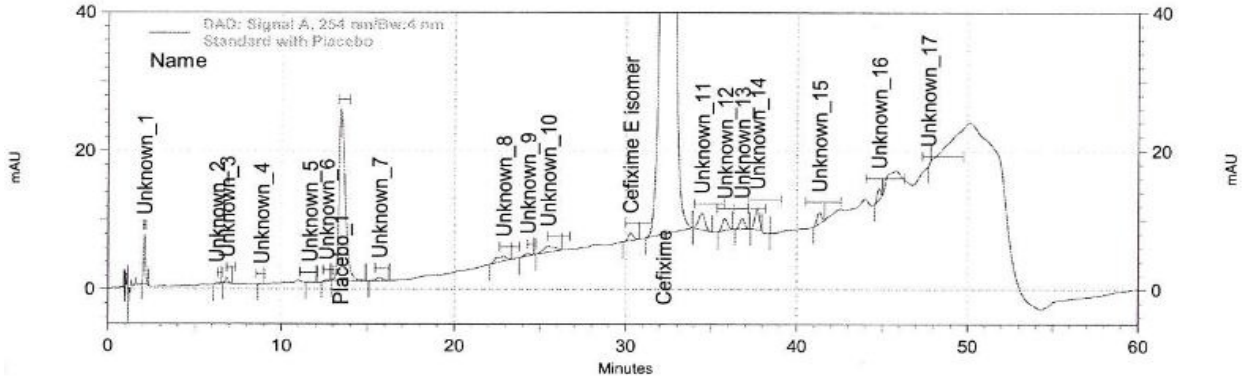
Chromatogram no: 28

A Representative chromatogram of Robustness High pH sample



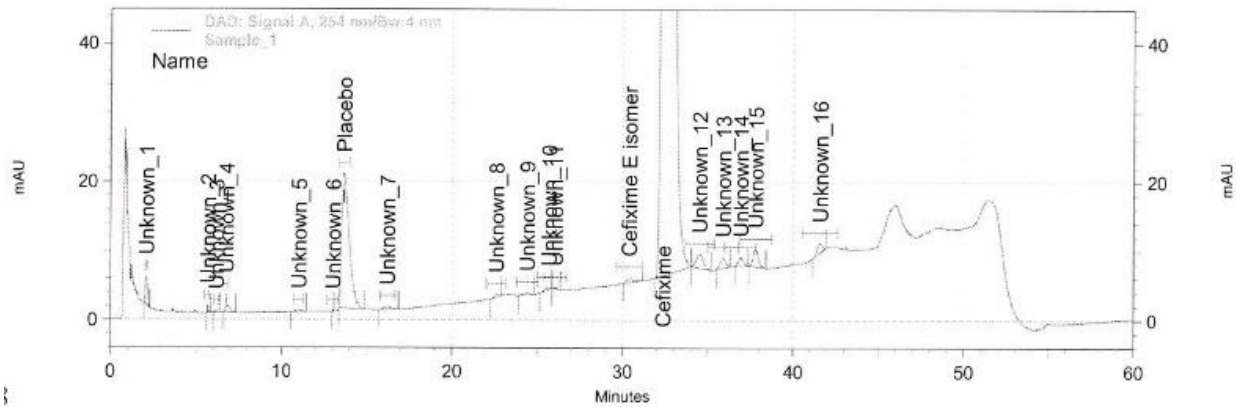
Chromatogram no: 29

Representative chromatogram of Acid stress sample



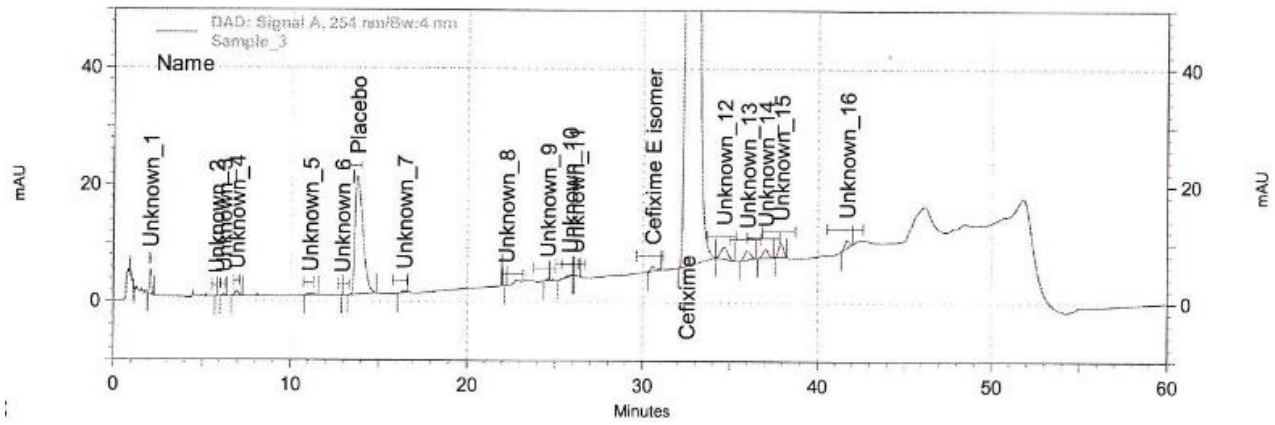
Chromatogram no: 30

Representative chromatogram of Base stress sample



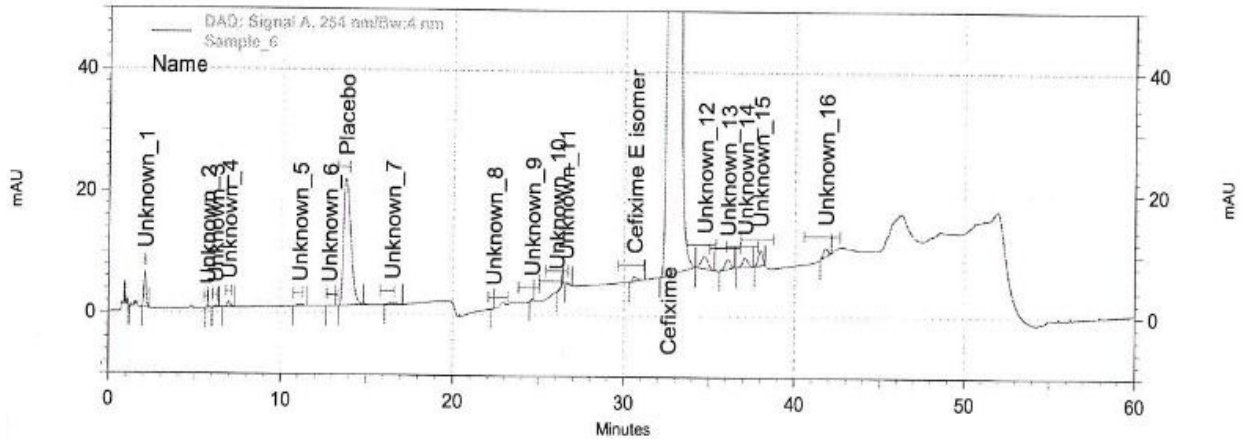
Chromatogram no: 31

Representative chromatogram of peroxide stress sample



Chromatogram no: 32

Representative chromatogram of thermal stress sample



10. RESULT AND DISCUSSION

VALIDATION OF THE METHOD

The solution stability studies were carried out at zero hour and after 24 hour, results were tabulated in table (1 and 2) The suitability of the system was studied by the values obtained for Theoretical plate, Resolution and tailing factor, %RSD of the chromatogram of standard drugs and presented in the table(3 and 4).

The selectivity of the method was revealed by the repeated injection of mobile phase and no interference was found and presented in Table (5 and 6)

The LOD and LOQ were calculated for Cefixime , it was presented in Table (7,8 and 9)

The limit of detection for cefixime was found to be 0.1µg/ml.

The Limit of Quantitation for cefixime was found to be 0.4µg/m

The linearity of proposed method were performed by using the concentration range of LOQ to 200% of standard concentration i.e 0.1 µg/ml to 0.6 µg/ml of *cefixime trihydrate* was presented in Table (10). The response factor, slope, intercept and correlation co-efficient were calculated. The slope, intercept, correlation co-efficient were found to be within the limit for cefixime . The calibration curves were plotted using response factor (Vs) concentration of standard solutions (fig: 02). The calibration graph shows that linear response was obtained over the range of concentration used in the procedure. These data demonstrates that the method have adequate sensitivity to the analytes. The range demonstrate that the method is linear outside the limits of expected use.

The Accuracy of the method was determined by recovery experiments. The recovery studies were carried out by preparing 4 individual samples with same procedure from the formulation and injecting. The percentage recovery and percentage relative standard deviation of the percentage recovery was calculated and presented in Tables (11). From the data obtained, added of standard drugs were found to be accurate.

The precision of the method was demonstrated by system and method precision. and intermediate precision of all solutions were injected into the chromatographic system performed by analyst 1 and analyst 2 The peak area and percentage relative standard deviation were calculated and presented in tables (12) & (13) ,The comparision of precision an intermediate precision presented in table (14)

The robustness of the method was studied by carrying out experiments by changing conditions discussed earlier. The response factors for these changed chromatographic parameters were almost same as that of the fixed chromatographic parameters Table (15to23) and hence developed method is said to be robust and ruggedness .The degradation of stress study of the product calculation presented in Table (24)

Validation protocal summary

Parameter	Experiment	Observation	Acceptance criteria
Solution stability	Bench top stability of standard solution	24 hours	The difference between initial and bench top stability sample for % of Relative standard deviation known impurity and Highest unknown impurity
	Bench top stability of Test solution	12 hours	
System suitability	% RSD Resolution	3.0	System NLT 2.0 suitability parameter should pass.
Specificity	Placebo and Blank, Impurity interference and Interference from Degradation products	Complies 1.0	There should not be any interference of blank, placebo peaks at the Retention Time (RT) of main peak and known impurity peaks. The Peak Purity should be not less than 0.9 in open lab software / purity angle should less than purity threshold for Empower Software.

Limit Of Detection And Limit Of Quantitation	Relative standard deviation method	19.3 % 2.0 %	The % Relative standard deviation for six replicate LOD level areas should be NMT 30.0%. The % Relative standard deviation for six replicate LOQ level areas should be NMT 10.0%.
Linearity and Range	Coefficient of correlation (r)	0.999 1.0 %	The Coefficient of correlation should not be less than 0.995. The Y intercept shall be ± 5.0% of the active response at 100% concentration.
Accuracy	% Recovery	Complies Complies	The % of Recovery at 50 % to 200% level should not be less than 90.0% and not more than 110.0%. The % Recovery at LOQ level should not be less than 80.0% and not more than 120.0%.
Precision	Method Precision	6.5% 3.7 % 6.9 %	The % RSD of known impurity, Highest unknown impurity and Total Impurities should not be more than 10.0.

	Intermediate Precision	4.0 % 1.9 % 3.2 %	The % RSD of known impurity, Highest unknown impurity and Total Impurities should not be more than 10.0. The overall % RSD of known impurity, Highest unknown impurity and Total Impurities obtained from method precision and intermediate precision results should not be more than 10.0
Robustness	Variation in column oven temperature	Complies	The over all % RSD of Known impurity, Highest unknown Impurity and Total Impurities obtained from method precision and robustness results should not be more than 10.0.
	Variation in flow rate	Complies	
	Variation in mobile phase pH	Complies	

Degradation Study	Acid stress	Complies	Total Impurities should be ±15% .The% RSD of peak responses between initial and bench top stability for diluted standard not more than 2.0.
	Base stress	Complies	Total Impurities should be ±15% .The% RSD of peak responses between initial and bench top stability for diluted standard not more than 2.0.
	Peroxide stress	Complies	
	Thermal stress	Complies	The Peak Purity should be not less than 0.9 in open lab software / purity angle should less than purity threshold for Empower Software.
	Peak purity	1.0	

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11. SUMMARY AND CONCLUSION

From the reported literature, there were few methods established for the determination of Cefixime was concluded that method reported for the Related substance of Cefixime oral suspension the above selected single component dosage form, which promote to pursue the present work. The scope and object of the present work is to develop and validate a new simple HPLC method for related substance of Cefixime Oral suspension dosage form.

In RP-HPLC method development, The Related substance of Cefixime oral suspension was carried out by using the Novapak C_{18} column (3.9 X 150 mm) with 4-micron particle size. Injection volume of 10µl is injected and eluted with the mobile phase phosphate Buffer, Acetonitrile with the gradient programme pH 6.5, which is pumped at the flow rate of 1.0 ml / min. Detection was carried out at 254 nm. Quantitation was done by calibration curve method with the above mentioned optimized chromatographic condition. This system produced symmetric peak shape, good resolution and reasonable retention times of cefixime E isomer and cefixime were found to be resolution is 3.0 and retension time is 30.8 and 33.02 minutes respectively.

The 0.4 µg/ml to 40 µg/ml of cefixime respectively. The slope intercept and correlation coefficient(s) were found to be, within the limit for which indicates excellent correlation factor Vs concentration of standard solutions.Precision of the developed methods was studied under system precision, method precision. The %RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust. The %RSD values for recovery percentage of Cefixime was found to be within the acceptable criteria. The result indicates satisfactory accuracy of method for estimation of the above mentioned drugs.Hence, the chromatographic method developed for Cefixime are rapid, simple, specific, sensitive, precise, Accurate. The RP-HPLC was simple and does not suffer from common excipients in pharmaceutical preparation and highly useful in the analysis of drugs in pharmaceutical formulation.

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