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Development and Validation of a RP-HPLA Method for Analysis of Anti-Inflammatory Drug Oxaceprol in API and Tablets

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ABSTRACT

Develop new simple, accurate and economical analytical methods for the estimation of Cobimetinib using RP-HPLC. Validate the proposed methods in accordance with USP and ICH guidelines for the intended analytical application i.e, to apply the proposed method for analyzing these drugs in tablet dosage form.

1. INTRODUCTION

1.1 Analytical chemistry

Analytical chemistry is the study of the separation, identification, and quantification of the chemical components of natural and artificial materials. Qualitative analysis gives an indication of the identity of the chemical species in the sample and quantitative analysis determines the amount of one or more of these components. The separation of components is often performed prior to analysis. In general terms, the pharmaceutical analysis comprises those procedures necessary to determine the "identity, strength, quality and purity" of such articles. For practical reasons, however it is proper to broaden the scope of this definition to include the analysis of raw materials. Analytical chemists in the pharmaceutical industry, as well as in those chemical industries that produce pharmaceutical raw materials must perform such analyses. The raw materials employed in the production of modern drugs, and the intermediates appearing during research, development and synthesis, involve thousands of diverse organic compounds. The pharmaceutical analyst must, therefore, have a firm grounding in basic organic analysis in addition to special skill in the quality evaluation of drug products. Unlike other major sub disciplines of chemistry such as inorganic chemistry and organic chemistry, analytical chemistry is not required to any particular type of chemical compound or reaction. Properties studied in analytical chemistry include geometric features such as molecular morphologies and distributions of species as well as features such as composition and species identity. To be effective and efficient, analyzing samples requires expertise in:

- The chemistry that can occur in a sample.
- Analysis and sample handling methods for a wide variety of problems (the tools-of- the-trade).
- Proper data analysis and record keeping.

Types:

Traditionally, analytical chemistry has been split into two main types:

Qualitative: qualitative analysis seeks to establish the presence of a given element or compound in a sample.

Quantitative: quantitative analysis seeks to establish the amount of given element or compound in a sample.

1.1.1 STEPS FOR ANALYTICAL DEVELOPMENT

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

1.1.1.1 Analyte standard characterization:

All information about the analyte i.e., physical and chemical Properties, toxicity, purity, hygroscopic nature, solubility and stability, the standard analyte (100% purity) is obtained, made an arrangement for the proper storage (refrigerator, desiccators and freezer). When multiple component analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.

1.1.1.2 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.

1.1.1.3 Literature search and prior methodology:

The information related to the analyte is surveyed. For synthesis, physical properties, 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.

1.1.1.4 Choosing a method:

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. 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.

1.1.1.3 Instrumental setup and initial studies:

The required instrumentation is setup. Installation, operational and performance qualifications of instrumentation using laboratory standard operating procedures (SOP) are verified. Always new solvents, filters are used, for example, method development is never started on a HPLC column that has been used earlier. The analyte standard in a suitable injection introduction solution and in known concentrations and solvents are prepared. If the sample is extremely close to the standard (e.g. bulk drug) then it is possible to start work with the actual sample.

1.1.1.3.1 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.

1.1.1.3.2 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.

1.1.1.3.3. 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.

1.1.1.3.4 Determination of percent recovery of actual sample and demonstration of quantitative sample analysis:

- 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 and shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty. 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 d is suitable for its intended applications. Quality is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods there can be no “second quality” in drugs.
- **Quality control:** is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production.
- **Physico-chemical methods** are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the physico-chemical methods, the most important are optical (Refractometry, Polarimetry, Emission, Fluorescence methods of analysis, Photometry including Photocolorimetry and Spectrophotometry covering UVVisible regions and Nephelometry or Turbidimetry) and chromatographic (Column, Paper, TLC, GLC, HPLC) methods. Methods such as Nuclear Magnetic Resonance and Para Magnetic Resonance are becoming more and more popular. The combination of Mass Spectroscopy with Gas Chromatography and Liquid Chromatography are the most powerful tools which are based on complex formation; acid- base, precipitation and redox reactions. Titrations in non- aqueous media and complexometry have also been used in pharmaceutical analysis. Instrumental methods of chemical analysis, instrumental method is a fascinating part of chemical analysis that interacts with all areas of chemistry and with many other areas of pure and applied sciences.

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

Precision Technologies and Instrumentation:

1. Spectrometric techniques

- Ultraviolet and visible Spectrophotometry
- Atomic Spectrometry (emission and absorption)
- Fluorescence and phosphorescence Spectrophotometry
- Infrared Spectrophotometry
- X-Ray Spectroscopy
- Raman Spectroscopy
- Radiochemical Techniques including activation analysis
- Nuclear Magnetic Resonance Spectroscopy
- Electron Spin Resonance Spectroscopy

2. Electrochemical Techniques

- Potentiometric
- Voltammetry
- Volta metric Techniques
- Aerometric Techniques
- Colorimetric Electro-gravimetric

3. Chromatographic Techniques:

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

4. Hyphenated Technique:

- 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)

Ultra- violet spectroscopy [5]

The term ultraviolet means **"beyond violet"** which is derived from the latin word **"**Ultra**"** meaning **"beyond".** The UV region of the electromagnetic spectrum ranges between 200-400 nm. The wavelength of ultraviolet radiation is shorter than that of visible light but no longer than x-ray. Ultraviolet region can be divided into following,

S/N	**Region**	**Wavelength (nm)**
1	Near ultraviolet region (NUV)	400-200 nm
2	Ultraviolet A(UVA) or long wave or black light	400-320 nm
3	Ultraviolet B(UVB) or medium wave	320-280 nm
4	Ultraviolet C(UVC)or shortwave	Below 280 nm

5	Far Ultra-violet (FUV) or vacuum ultra-violet (VUV)	200-10 nm

Table 1: Types ultraviolet regions with their wavelength ranges.

Beer's law:

According to the Beer's law, "when a beam of monochromatic radiation passes through an absorbing medium, the intensity of incident radiation decreases exponentially with increase in the concentration of the absorbing medium." In other words, absorbance is directly proportional to the concentration of the absorbing substance.

$$I = It.\ e - kc$$

Lambert's law:

According to lambert's law, "the rate of decrease in the intensity of the incident radiation

(I) with the thickness of the medium (t) is directly proportional to the intensity of the incident light x''. Combined equation of Beer- Lambert's law:

$$A = act$$

Where;

A- Absorbance a - Absorptivity

C – Concentration

t- path length or thickness

Types of transitions:

σ→σ* Transitions: This occurs in saturated compounds which do not contain lone pair of electrons. Examples: Methane, Propane.

π→π* Transitions : This occurs in unsaturated molecules. Examples: Alkenes, Alkynes.

n→σ* Transitions : This occurs in saturated compounds which contain lone pair of electrons. Examples: Ethers, Aldehydes.

n→π* Transitions : This occurs in unsaturated molecules having atoms like Sulphur, oxygen, nitrogen, Ketones.

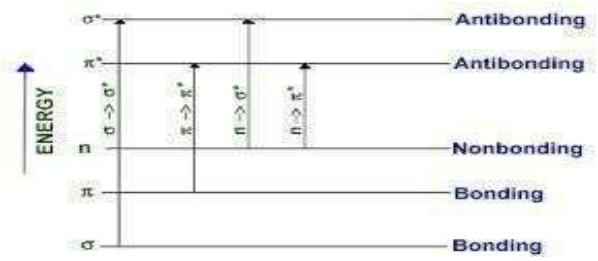


Figure 1: Electronic transitions

2. AIM AND OBJECTIVES

An extensive literature survey was carried out for cobimetinib (cotellic) and all its related substances. The present study was undertaken with an objective of developing a rapid, simple, cost effective, gradient stability indicating RP- HPLC method for the estimation of related substance and impurities in orally tablets.

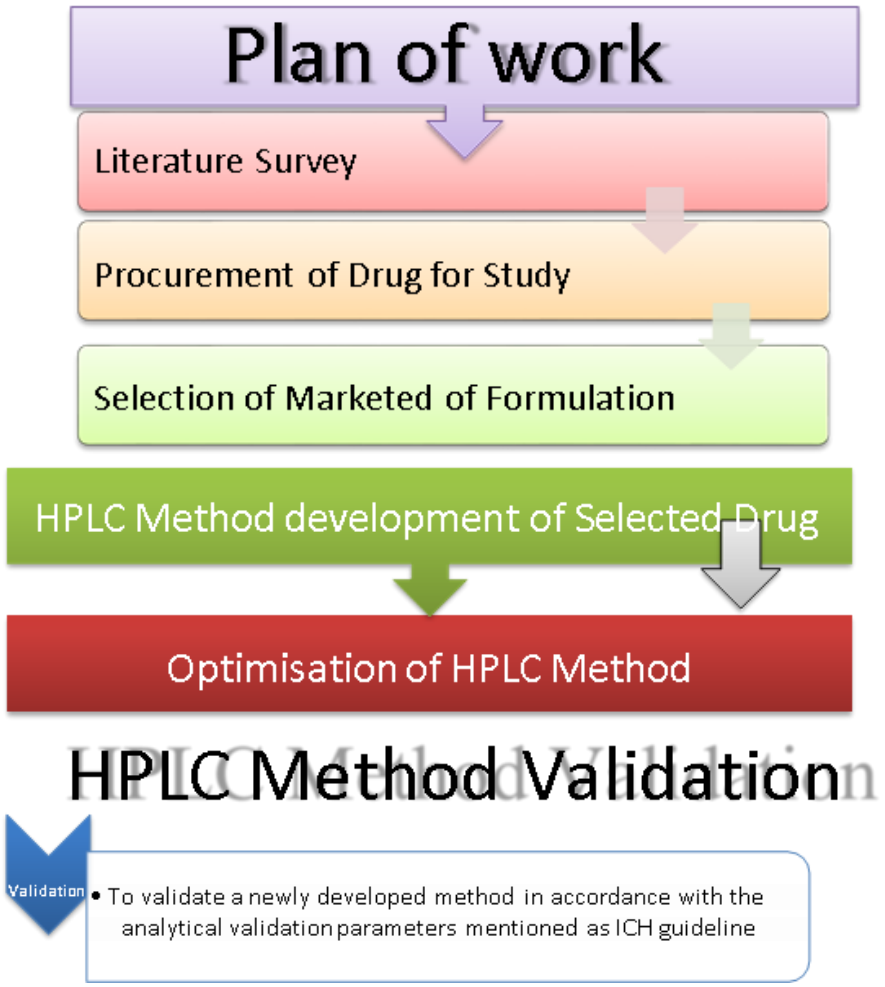
Cobimetinib (cotellic) belong to the class of antineoplastic agents. They are used to treat Melanoma a type of skin tumour that is produced in cells producing pigments, melanocytes mutate and turn out cancerous. BRAF is a constituent of the mitogen- activated protein kinase (MAPK) signalling pathway, which serves to activate downstream MEK, and is one of the most commonly mutated oncogenes in human tumours. Indeed, BRAF V600 mutations are present in approximately 40% of metastatic melanoma tumours.

The present study is to make an attempt to establish sensitive, economic and accurate methods for the estimation of Cobimetinib in pure and pharmaceutical dosage form. The proposed methods will be validated as per ICH guidelines. Literature survey reveals that only few analytical methods have been reported for the estimation of Cobimetinib in bulk drug and pharmaceutical formulations. Hence, an attempt has been made to develop simple, accurate, sensitive, rapid and economic method for the estimation of Cobimetinib in pharmaceutical dosage forms using UV–Visible spectrophotometry and High Performance Liquid Chromatography techniques. These methods can also be applied for estimation of pure drug.

3. PLAN OF WORK

An attempt was made in a stepwise manner to develop a simple, reproducible and efficient analytical method using RP-HPLC for estimation of Cobimetinib (cotellic) in API and tablet form.

Following stepwise protocol was followed:



4. DRUG PROFILE:

Osteoarthritis is a degenerative synovial joint disease that affects the cartilage and bone of both major and small joints, impairing one's capacity to work and, depending on the joints involved, daily activities. The most common symptoms are joint pain and stiffness, which can progress to joint dysfunction, deformities, and muscular weakness. It is the most common type of arthritis, with an estimated 250 million people suffering from osteoarthritis of the knee alone across the world. It is thought to be the fourth greatest cause of disability in Asia. However, osteoarthritis being a widespread disease and joint surgery being a later option available at only a few specialized centers, the quest for new drugs for osteoarthritis must continue. The search for a truly disease modifying anti-osteoarthritis drug remains elusive

1 . Oxaceprol is an amino acid derivative, which has been used for decades for the symptomatic treatment of degenerative and inflammatory joint disease in Europe .Oxaceprol has anti-inflammatory and analgesic efficacy comparable to the conventional non-steroidal anti-inflammatory drugs (NSAIDs) but has a different mode of action. Instead of inhibiting the synthesis of prostaglandins oxaceprol prevents leukocyte infiltration into the joints, thus inhibiting an early step of inflammatory cascade and presenting a novel class of anti-inflammatory agents2

INDICATIONS: Oxaceprol drug is having anti-inflammatory pharmacological activity which is mainly used in the treatment of osteoarthritis/ disorders of joint and connective tissue inflammation. This drug mainly acts by reducing leukocyte accumulation in joints and thereby inhibits the inflammation SIDE EFFECTS:

- Rashes on the skin.
- Abdominal Pain

- Constipation
- Epigastria pain

5. REVIEW OF LITERATURE

• **JifengGu et al.,** Has reviewed that sensitive method for the quantification of oxaceprol in rat plasma using high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed. Sample pretreatment involved a simple protein precipitation by the addition of 60 μL of acetonitrile–methanol (1:2, v/v) to 20 μL plasma sample volume

• **G. Herrmann et al.,** Has reviewed that the therapeutic equivalence and safety of treatment for 21 days with 400 mg t.i.d. oxaceprol (n = 132) and 50 mg t.i.d. diclofenac (n = 131) were assessed in a multicentre, randomized, double-blind study of a mixed population of patients with osteoarthritis of the knee and/or hip. In a per-protocol analysis of efficacy, the mean Lequesne index decreased by 2.5 points in the oxaceprol group (n = 109) and by 2.8 points in the diclofenac group (n = 109). The 95% confidence interval for the end-point difference revealed therapeutic equivalence. This was confirmed by assessments (visual analogue scale) of pain at rest, weight-bearing pain, pain on standing and pain on movement, all of which decreased to a similar extent under both treatments

• **Ionac M et al.,** Has reviewed that Oxaceprol, an established therapeutic agent for osteoarthritis, had no effect on macrophage prostaglandin E2 release in vitro and inhibited carrageenan paw oedema at high doses (18– 150 mg/kg p.o.). In the same dose range, oxaceprol was comparable to indomethacin (3 mg/kg p.o.) as an inhibitor of yeast hyperalgaesia and at 6– 50 mg/kg/day p.o. had a mild, variable inhibitory effect on cotton pellet granuloma formation

• **Harpreet Singh Pawar et al.,** Has reviewed that the duration and dose dependent side effects of conventional intra-articular corticosteroid treatment in osteoarthritis (OA) like cartilage damage and chondrocyte toxicity warrant the search for alternative therapeutics. Oxaceprol, a 6 Recognized oral therapeutic agent for osteoarthritis is yet to be explored for its intra-articular route of administration confirming better safety profile. In this study, a comparative evaluation of intra-articular oxaceprol and corticosteroid is carried out in osteoarthritis rabbit model. Osteoarthritis was induced by monosodium iodoacetate in rabbits

• **Seher Karsli-Ceppioglu et al.,** Has reviewed that Oxaceprol is well-defined therapeutic agent as an atypical inhibitor of inflammation in osteoarthritis. In the present study, we aimed to develop and characterize oxaceprol- loaded poly-lactide-co-glycoside (PLGA) nanoparticles for intraarticular administration in osteoarthritis. PLGA nanoparticles were prepared by double-emulsion solvent evaporation method

• **Kaushik Mukhopadhyay et al.,** Has reviewed that to assess efficacy and safety of oxaceprol, a hydroxyproline derivative with putative mechanism of action different from traditional nonsteroidal anti-inflammatory drugs, in symptomatic knee osteoarthritis, in comparison to tramadol.

• **Andreas Veihelmann et al.,** Has reviewed that Oxaceprol (N-acetyl-Lhydroxyproline), an atypical inhibitor of inflammation, is an established drug for joint disease without serious side-effects. Recent studies have emphasized that oxaceprol has an effect on the microcirculation. Since the exact mechanism of action remains unclear, the aim of our study was to investigate the leukocyte-endothelial cell interactions in oxaceprol-treated mice with antigen-induced arthritis (AiA) using intra vital microscopy

6. METHODOLOGY

6.1 REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In the present investigation, we have developed a simple and sensitive. RP-HPLC method for quantitative estimation of cobimetinib fumerate bulk drug.

Apparatus and software**:**

The Agilent 1120 Compact LC HPLC (4.6 X 250 mm, 5µm), mobile phase consisting of methanol: Phosphate buffer at pH 3 was used. Flow rate was at 1m1 /min and the column was maintained at ambient temperature condition was used in HPLC analysis. The detector using photo diode array was set at a wavelength of 285 nm. HPLC grade water obtained by using Direct-Q water purification system (Millipore, Milford, USA) was used in HPLC study. Due to its speed accuracy, this method can be used for estimation and analysis of Cobimetinib in active pharmaceutical ingredient and pharmaceuticals.

Collection of reagents and solvents:

Each tablet consisted of 22 mg of cobimetinib fumarate,which corresponds to 20 mg of cobimetinib free base as active ingredient. HPLC grade Methanol (Merck), Analytical column C18 (4.6 X 150 mm, 5µm), mobile phase consisting of methanol: Phosphate buffer at pH 3 in the ratio of 70:30%v/v was used. Flow rate was at 1ml / min and the detector using photo diode array was set at a wavelength of 285 nm. HPLC grade water obtained by using Direct-Q water purification system (Millipore, Milford, USA) was used in HPLC study. Due to its speed accuracy, this method can be used for estimation and analysis of Cobimetinib in active pharmaceutical ingredient and pharmaceuticals.

6.2 ANALYTICAL METHODS DEVELOPMENT

6.2.1 OPTIMIZATION OF UV CONDITIONS

from 200-400 nm of cobimetinib standard solutions. By observing the spectra of standard Initially method development work was started by taking UV - visible spectra solutions 2. max 265, 370 nm were taken for trials to develop UV method. The isobestic point of the combination was found to be 285 nm.

6.1.1 METHOD DEVELOPMENT TRIALS

The objective of this experiment was to optimize the assay method for estimation cobimetinib based on the literature survey made, so here are several trials were conducted for method development to optimize various parameters.

TRAIL1

CHROMATOGRAPHIC CONDITIONS:

Table No. 4: Chromatographic Condition: Trail-1

Mobile Phase	Methanol : phosphate buffer
	70 : 30
Flow Rate	1 ml/min
Column	Agilent Eclipse plus C_8 Column (4.6 X 250 mm, 5 µm)
Detector Wavelength	285 nm
Injection Volume	20 µl

TRAIL 2

CHROMATOGRAPHIC CONDITIONS:

Table No. 5: Chromatographic Condition: Trail-2

Mobile Phase	Phosphate buffer : : Methanol
	50 : 50
Flow Rate	1 ml/min
Column	Agilent Eclipse plus C_8 Column (5 µm 4.6x250 mm)
Detector Wavelength	285 nm
Injection Volume	20 µl

6.1.1 METHOD DEVELOPMENT TRIALS

The objective of this experiment was to optimize the assay method for estimation cobimetinib based on the literature survey made, so here are several trials were conducted for method development to optimize various parameters.

TRAIL1

CHROMATOGRAPHIC CONDITIONS:

Table No. 4: Chromatographic Condition: Trail-1

Mobile Phase	Methanol : phosphate buffer
	70 : 30
Flow Rate	1 ml/min

Column	Agilent Eclipse plus C_8 Column (4.6 X 250 mm, 5 µm)
Detector Wavelength	285 nm
Injection Volume	20 µl

TRAIL 2

CHROMATOGRAPHIC CONDITIONS:

Table No. 5: Chromatographic Condition: Trail-2

Mobile Phase	Phosphate buffer : : Methanol
	50 : 50
Flow Rate	1 ml/min
Column	Agilent Eclipse plus C_8 Column (5 µm 4.6x250 mm)
Detector Wavelength	285 nm
Injection Volume	20 µl

6.3 METHODOLOGY

6.3.1 BUFFER PREPARATION

0. IN Potassium Dihydrogen orthophosphate and methanol were prepared and pH 5.5 was adjusted with dilute ortho phosphoric acid.

6.3.2 PREPARATION OF MOBILE PHASE

Buffer and methanol were mixed at 50:50 ratios, sonicated the resulting solution and degassed it using vacuum filtration through 0.4µ membrane filter.

6.3.3 DILUENT PREPARATION

Mobile phase was used as a Diluent.

6.3.4 STANDARD STOCK SOLUTION PREPARATION

About 25.0 mg of Cobimetinib working standards were weighed and transferred into 50 mL volumetric flask, 25 mL of diluent was added and sonicated to dissolve and diluted to volume with diluent.

6.3.5 STANDARD PREPARATION

1 mL of standard stock solution was transferred into 10 mL volumetric flask and diluted to volume with diluent.

6.3.6 SAMPLE PREPARATION

20 mg were weighed, average weight was calculated and the tablets were crushed into fine powder in a mortar with pestle separately. Accurately weighed and transferred sample quantitatively equivalent (Average Weight) to 20 mg of cobimetinib in to 50 mL volumetric flask 25 mL of diluents was added sonicated to dissolve for 10 minutes and diluted to volume with diluent Further filtered the solution through filter paper and Diluted ml of filtrate to 10 ml with mobile phase.

ASSAY SYSTEM CHROMATOGRAM

- 20L of the Blank solution was injected into chromatographic was recorded of the standard solution was injected into chromatographic system.
- 20ul were recorded and peak areas were measured chromatograms.
- 20ul of the sample solution was injected into chromatographic system, chromatograms were recorded and peak areas were measured.

ACCEPTANCE CRITERIA

- % RSD for the peak areas of responses of five replicate injections of standard solutions should be not more than 2.0 %.
- The number of theoretical plates should be not less than 2000.
- The Tailing factor (T) should be not more than 2.0

METHOD VALIDATION

Validation documentation evidence, which provides a high degree of establishing that specific process, will consistently produce a product meeting its assurance and quality attributes, specification predetermined range from:

6.3.7 LINEARITY CONCENTRATION

A series of solutions of working standard were prepared in the 25 % to 150 % of test concentration to demonstrate linearity for assay and injected into chromatographic system. The graph was plotted between standard area and concentration.

STANDARD STOCK SOLUTION

About 25 mg of cobimetinib working standards were weighed and transferred into 50 mL volumetric flask, 25 mL of diluent was added and sonicated for 10 min to dissolve completely and volume was made up to the mark with diluent and filtered through 0.45u filter paper.

A. PREPARATION OF LEVEL 1 (25 %):

0.25 mL of stock solution was pipetted into a 10 mL volumetric flask and diluted up to the mark with diluent.

B. PREPARATION OF LEVEL 2 (50 %):

0.5 mL stock solution was pipetted into a 10 mL volumetric flask and diluted up to the mark with diluent.

C. PREPARATION OF LEVEL 3 (75 %):

The 0.75mL of stock solution was pipetted into a 10ml volumetric flask and diluted up to Mark with Diluent.

D. PREPARATION OF LEVEL 4 (100 %):

1.0 mL of stock solution was pipetted into a 10 mL volumetric flask and diluted up to the mark with diluent.

E. PREPARATION OF LEVEL 5 (125%):

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

F. PREPARATION OF LEVEL 6 (150 %):

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

Linear solution (%)	**Stock solution taken in (ml)**	**Diluted to volume (ml) with diluent**
25	0.25	10
50	0.50	10
75	0.75	10
100	1.00	10
125	1.25	10
150	1.50	10

TABLE 8 : PREPARATION AT 25% TO 150% LEVEL

PROCEDURE

Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X - axis concentration and on Y - axis Peak area) and calculate the correlation coefficient.

ACCEPTANCE CRITERIA

Correlation coefficient should be not less than 0.999.

6.3.8 PRECISION

The precision of an analytical method is a measure of the random error and is between replicate measurements of the same sample. It is defined as the agreement of variation (° C) or relative standard deviation coefficient expressed as the percentage (RSD) of the replicate measurements

% CV = standard deviation/Mean X 100

A. PROCEDURE FOR SYSTEM PRECISION STANDARD STOCK SOLUTION

About 25 mg of Cobimetinib working Standards were weighed into 50 mL volumetric flask, 25 mL of diluent was added and sonicated for 10 min to dissolve completely and volume was made upto the mark with diluent and filtered through 0.45 u filter paper.

PREPARATION OF SOLUTION

1 ml of standard stock solution was diluted with 10 mL of diluent the above solution was injected six times.

B. PROCEDURE FOR METHOD PRECISION STANDARD STOCK SOLUTION

About 25.0 mg of cobimetinib working standards were weighed into 50 mL volumetric flask, 25 ml of diluent was added and sonicated for 10 min to dissolve completely and volume was made up to the mark with diluent and filtered through 0.45 u filter paper.

PREPARATION OF SOLUTION

1 ml of standard stock solution was diluted with 10 mL Six solutions were prepared and each solution was injected

ACCEPTANCE CRITERIA

The % of RSD for Area and RT from Repeated injections should not be more than 2%.

6.3.8 ACCURACY

The accuracy of the test method is demonstrated by % of recovery. The sample preparations are spiked with known amount of standard at three concentration levels and each is injected three times (Like 80 % 100 % and 120%).

PREPARATION OF STANDARD STOCK SOLUTION

About 25 mg of Cobimetinib working standards were transferred into 50 ml volumetric flask, 25 mL of diluents was added and sonicated for 10 min to dissolve completely and volume was made up to the mark with diluent and filtered through 0.45 u filter paper.

SAMPLE STOCK SOLUTION PREPARATION OF (50 %) ACCURACY

1 ml of sample stock solution was taken into 10 mL volumetric flask; 1 mL of standard stock solution was added to it and diluted up to the mark with diluent.

PREPARATION OF (100 %) ACCURACY

I ml of sample stock solution was taken into 10 mL volumetric flask; 3 mL of standard stock solution was added to it and diluted up to the mark with diluent.

PREPARATION OF (150 %) ACCURACY

1 ml of sample stock solution was taken into 10 ml volumetric flask: 5 ml of standard stock solution was added to it and diluted up to the mark with diluent.

6.3.9 ROBUSTNESS

The robustness of test method is demonstrated by carrying out intentional method Variations like mobile phase flow changes, mobile phase compositions and column oven temperature variations etc.

EFFECT OF FLOW RATE VARIATION

Standard solution prepared as per test method was injected into chromatographic system with 12 mL/ min of flow rate System suitability parameters were evaluated.

6.3.10 SPECIFICITY

Standard and test samples were compared for the Retention times obtained from working identification.

STANDARED SOLUTION PREPARATION

A. COBIMETINIB (COTTELLIC)

About 25 mg of cobimetinib working standard was weighed and transferred into 50 mL volumetric flask, 25 mL of diluents was added and sonicated to dissolve and diluted to volume with diluent. Further 1 mL of above solution was transferred into 10mL volumetric flask and diluted to volume with diluent.

B. COBIMETINIB (COTELLIC)

About 6.25 mg of cobimetinib working standard was weighed and transferred into 50mL volumetric flask, 25 mL of diluents was added and sonicated to dissolve and diluted to volume with diluent. Further 1 mL of above solution was transferred into 10 mL volumetric flask and diluted to volume with diluent.

MIXED STANDARD

1 mL of cobimetinib Standard stock solution were transferred into 10 mL volumetric flask and diluted to volume with diluent.

SAMPLE SOLUTION

Assay solution was used as sample preparation.

PLACEBO PREPARATION

Placebo solution was prepared same as like sample.

BLANK PREPARATION

Diluent was used as Blank solution preparation.

PROCEDURE

Blank Placebo Solution mixed standard and sample Solution were injected.

ACCEPTANCE CRITERIA: RT of sample should match with the standard RT.

6.4.6 Ruggedness (Intermediate Precision): The ruggedness of test method is demonstrated by carrying out precision studies with different analysts and on different days.

A. Procedure for System Intraday Precision:

Standard Stock Solution:

About 20.5 mg of Cobimetinib working standard were transferred into 50 ml of diluent was added and sonicated for 10 min to dissolve completely and volume was made up to the mark with diluent and filtered through 0.45 µ filter paper.

Preparation of Solution:

1 ml of standard stock solution was diluted with 10 ml of diluent. The above solution was injected five times.

B. Procedure for Method Interday Precision:

Standard Stock Solution:

About 20.5 mg of Cobimetinib working standard were transferred into 50 ml of diluent was added and sonicated for 10 min to dissolve completely and volume was made up to the mark with diluent and filtered through 0.45 µ filter paper.

Preparation of Solution:

1 ml of standard stock solution was diluted with 10 ml of diluent. The above solution was injected five times.

Acceptance Criteria: The % of RSD for Area and RT from repeated injection should not be more than 2.0%.

Limit of detection and limit of quantification

LOD and LOQ were calculated according to ICH recommendations where the approach is based on the signal –to-noise ratio. Chromatogram signals obtained with known low concentrations of analytes was compared with the signals of the blank samples.

Limit of detection is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. Limit of quantitation is determined by the analysis of samples with known concentrations of analyte

and by establishing the minimum level at which the analyte can be reliably quantitate. The LOQ was calculated based on the LOD strength, the LOD values were multiplied by three times to get LOQ

7. RESULT

7.1 Optimization of UV Method Development:

In HPLC method, HPLC conditions were optimized to obtain, an adequate separation of eluted compounds, initially, various mobile phase composition was tried to elute title ingredient. Mobile phase and flow rate selection was based on peak parameters (height, capacity, theoretical plates, tailing or symmetry factor), run time, resolution.

S.No	Parameters	Estimation of Cobimetinib
1	Mobile phase optimized	Methanol and Phosphate buffer (50:50)
2	Stationary phase	Agilent Eclipse plus C8 5µm 150 X 4.6 mm
3	Flow rate (ml/min)	1.0 ml
4	Column Temperature OC	250c
5	Volume of Injection (µl)	10
6	Detection Wavelength (nm)	285
7	Retention time Rt	5.323 min

Table 9: Optimization of UV Method Development:

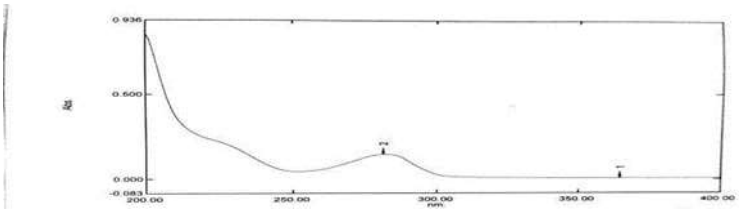


Figure No. 10: cobimetinib UV-Spectrum

Observation:

Sr No.	Wavelength	Absorbance
1	268 .00	0.498

TRAIL 1

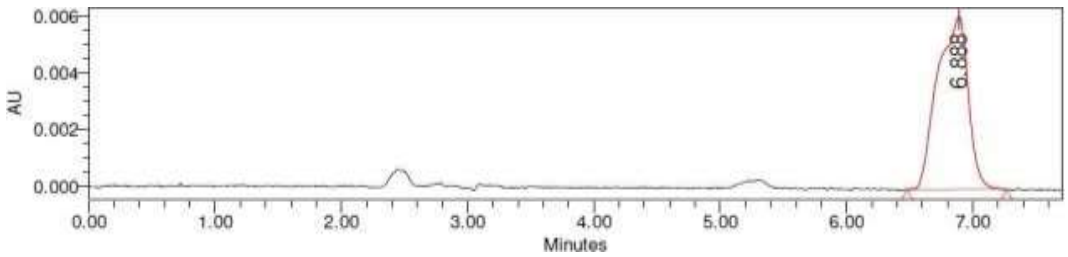


Figure NO.11 Observation

TRAIL 2

Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
6.888	102706	-	0.82	3598

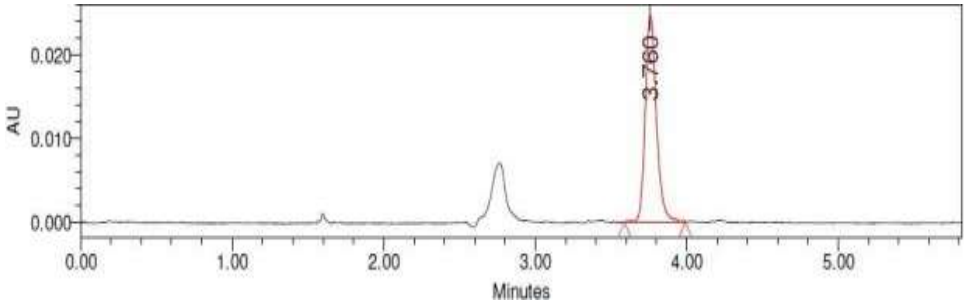


FIGURE NO. 12

Observation

Remarks: Two peaks eluted but first peak was not properly eluted

TRAIL 3

Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
3.760	125010	-	1.22	13566

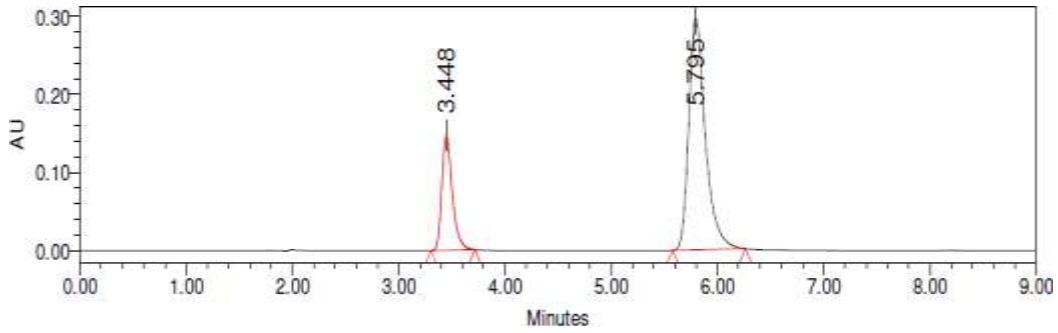


FIGURE NO. 13

Observation

Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
3.448	1011473	23.94	146967	-	1.36	6117
5.795	3214256	76.06	296834	10.07	1.41	7141

Remarks: Two peaks with all satisfied system suitability values eluted and these conditions are choosen

7.1 ASSAY:

CHROMATOGRAMS

After several trials with the different combination and ratio of solvents, the mobile phase methanol: phosphate buffer PH 3 (50:50v/v). Retention time (Rt) 5.32 min for Cobimetinib. Wavelength was selected by scanning the standard drug over a wide range of wavelength 200 nm to 400 nm. The component shows reasonably good response and maximum peak at 285nm.

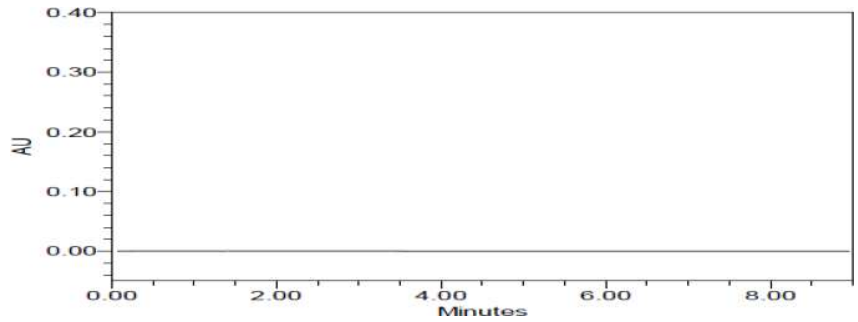


Figure No. 14: Chromatogram of blank

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Sample (cotellic) standard Assay-1	Cobimetinib	2.323	577447	3545	1.056

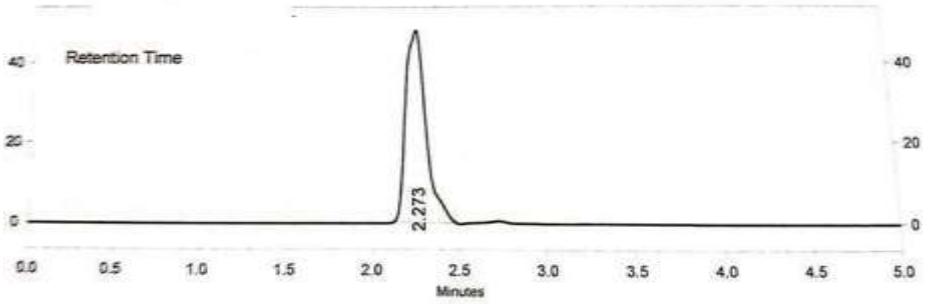


Figure No.16: Chromatogram 1 of cobimetinib sample solution at 285 nm

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Sample (cotellic) sample Assay-2	Cobimetinib	2.273	555447	3519	1.059

Assay of cobimetinib.

Sr No.	Cobimetinib		
		RT	**Area**
1	**Standard 1**	2.323	577447
1	**Assay sample 1**	2.273	555447

7.2 VALIDATION OF ANALYTICAL METHOD:

7.1 Selectivity:

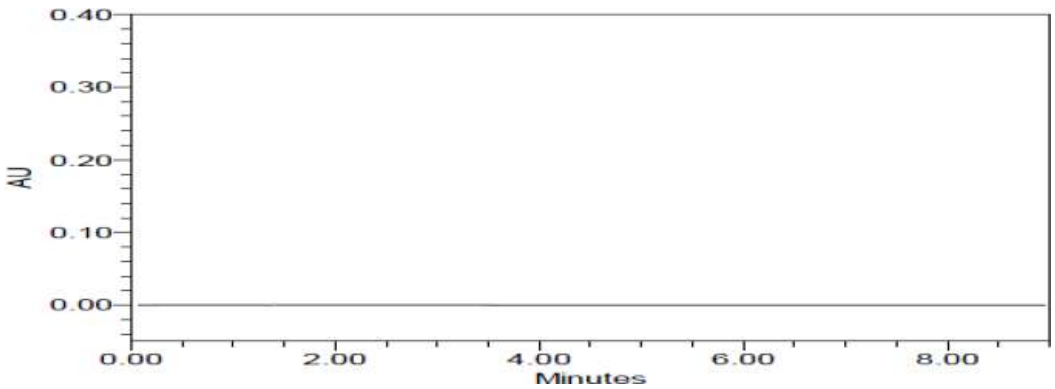


Figure 17: Chromatogram showing blank.

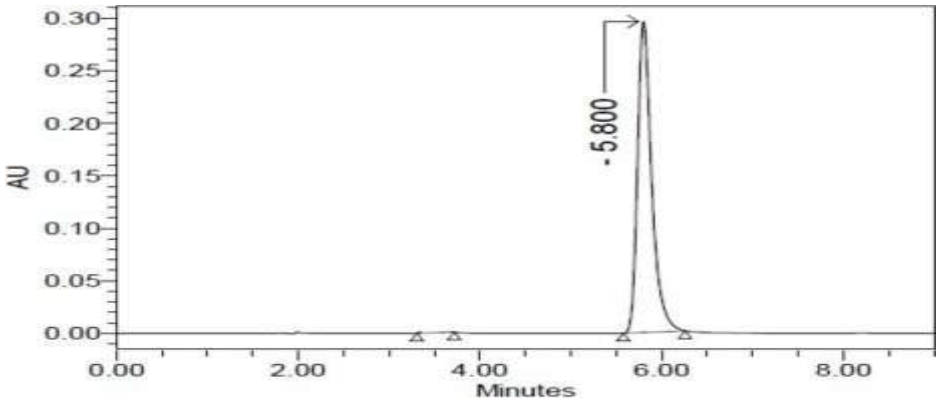


Figure 18: Chromatogram showing standard

Observation:

Title	**Name**	**Ret Time**	**Area**	**USP Plate Count**	**Tailing Factor**
selectivity	Cobimetinib	5.800	232788	3496	1.085

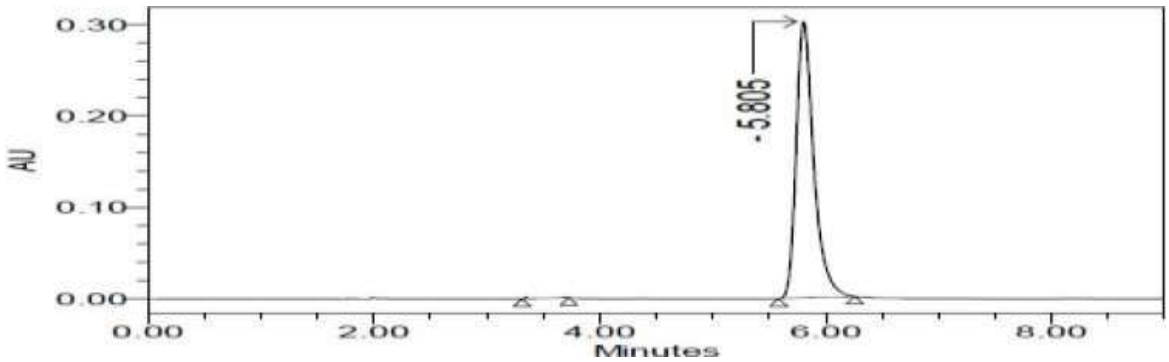


Figure 19: Chromatogram showing sample

Observation

Title	**Name**	**Ret Time**	**Area**	**USP Plate Count**	**Tailing Factor**
selectivity	Cobimetinib	5.805	238978	3496	1.085

7.2 Linearity and range:

For linearity analyte concentration for cobimetiniib fumerate taken across 50 µg/ml to 150 µg/ml of cobimetinib fumerate. They were prepared using HPLC grade methanol as solvent. Then tested at 268 nm. Absorbance is plotted graphically as a function of analyte concentration. By using the working standard, aliquots of 50 µg/ml, 75 µg/ml, 100µg/ml, 120 µg/ml, were prepared with Methanol. Five dilutions of each of the above mentioned concentrations were prepared separately and from these six dilutions, 20 µL of each concentration were injected into the HPLC system. Then their chromatogram was recorded. Peak areas were recorded for all the peaks and a standard calibration curve of peak area against concentration was plotted,

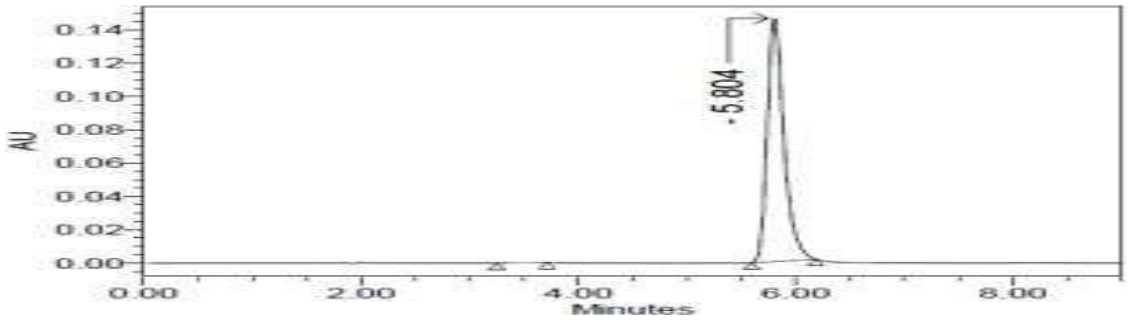


Figure 20: Chromatogram showing linearity level-1(50)

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Linearity	Cobmetinib	5.804	232718	3496	1.086

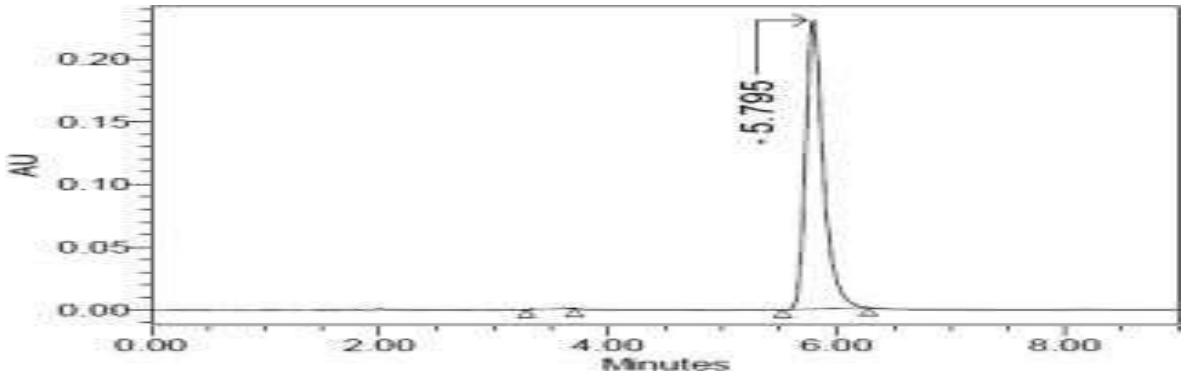


Figure 21: Chromatogram showing linearity level-2(75)

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Linearity	Cobimetinib	5.795	406835	34886	1.089

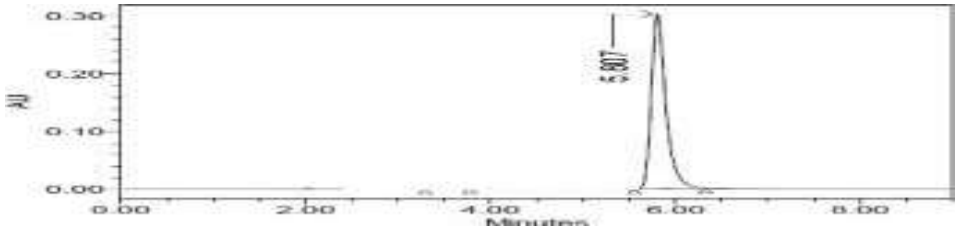


Figure 22: Chromatogram showing linearity level-3(100)

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Linearity	Cobimetinib	5.807	498772	3490	1.090

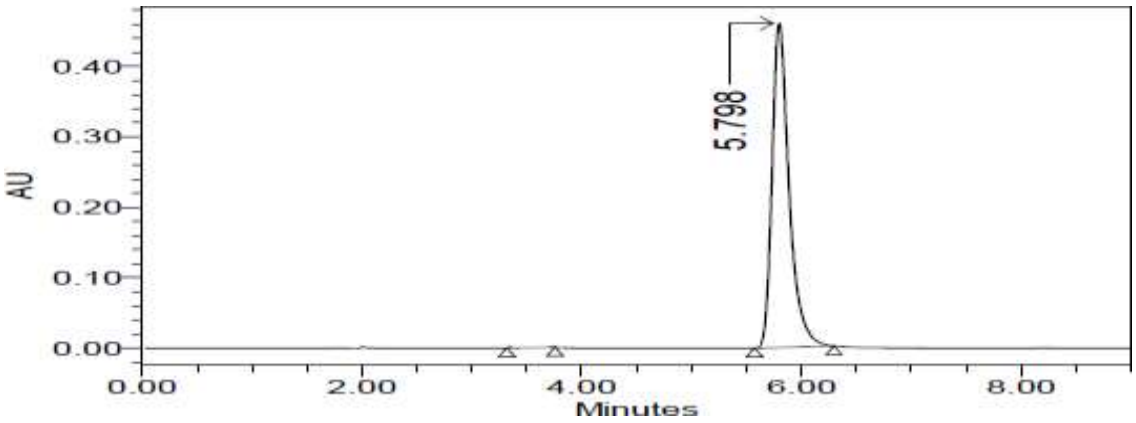


Figure 23: Chromatogram showing linearity level-3(120)

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Linearity	Cobimetinib	5.798	647610	3499	1.098

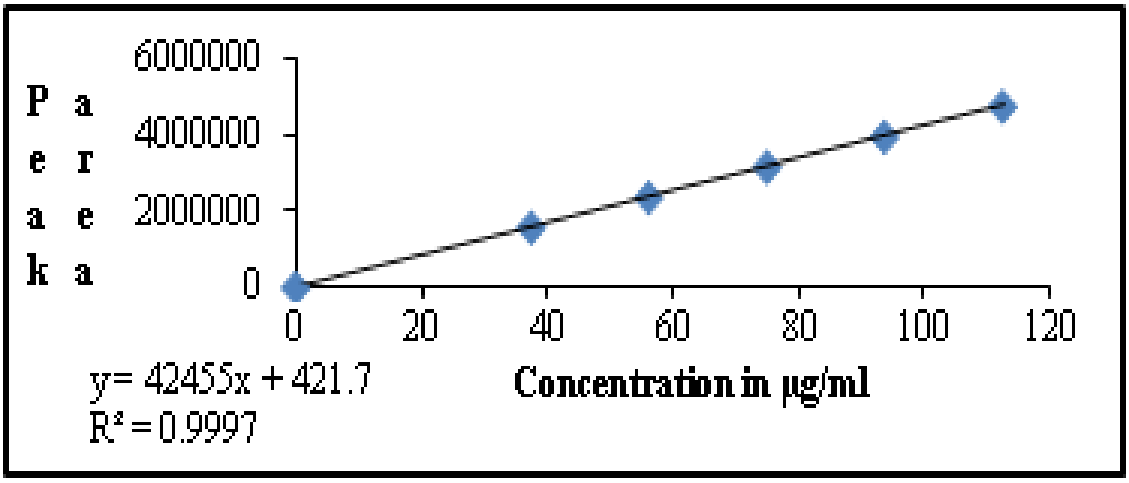


Figure 24: Cobimetinib linearity confirmation curves

Table No. 10: Observations for linearity

Sr. No	Level	Concentration mg/ml	Retention time(min.)	Area	RSD
1.	50	0.050	5.804	232718	0.63
2.	75	0.075	5.795	406835	0.23
3.	100	0.100	5.807	498772	0.82
4.	120	0.120	5.798	647610	0.19

Table No. 11: Observation Data

Level %	Conc mg/ml	Area
50	0.050	232718
75	0.075	406835
100	0.100	498772
120	0.120	647610

7.2 Precision:

Precision of the analytical method was studied by analysis of multiple sampling of homogenous sample

7.2.1 System Precision:

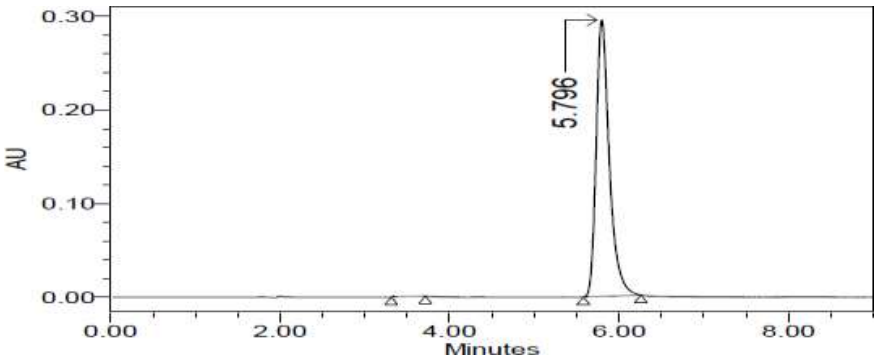


Figure 25: System precision chromatogram Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
precision	Cobimetinib	5.796	318739	3499	1.093

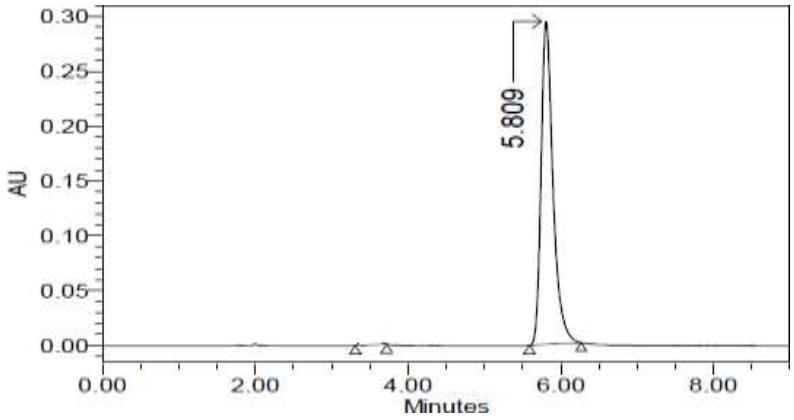


Figure 26: System precision chromatogram (2)

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
precision	Cobimetinb	5.809	432718	3496	1.085

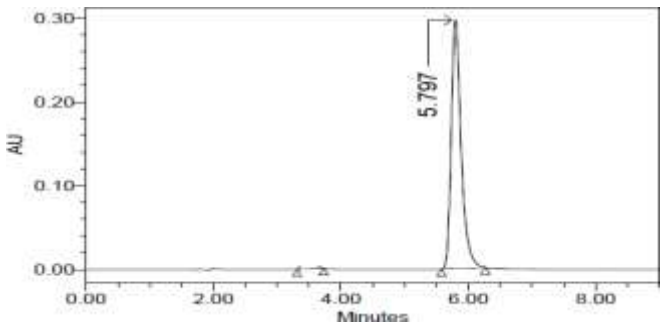


Figure 27: System precision chromatogram (3)

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
precision	Cobimetini B	5.797	417231	3678	1.095

Table 12: System precision Data:

Sr. No	RT	STD Area
1	5.796	318739
2	5.809	432718
3	5.797	417231
Average	5.800	890534
Std. Dev	0.0008	485.118
% RSD	0.05	0.13

7.3.1 Method System Precision:

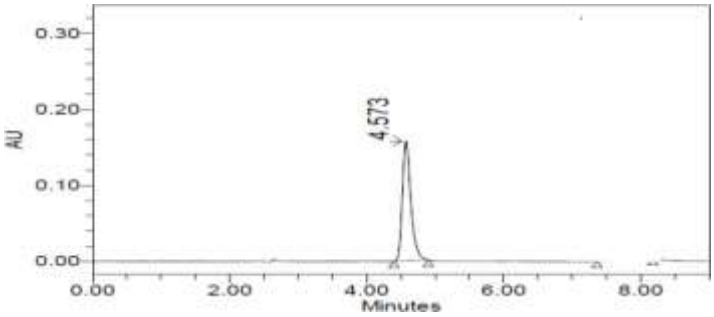


Figure 28: Method precision chromatogram 1

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Method precision 1	Cobimetinib	4.573	567890	3656	1.84

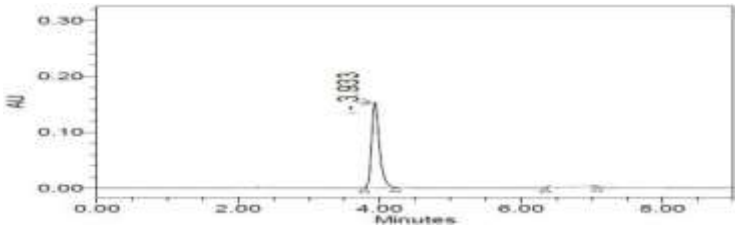


Figure 29: Method precision chromatogram 2

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Method precision 2	Cobimetinib	3.933	456732	3646	1.87

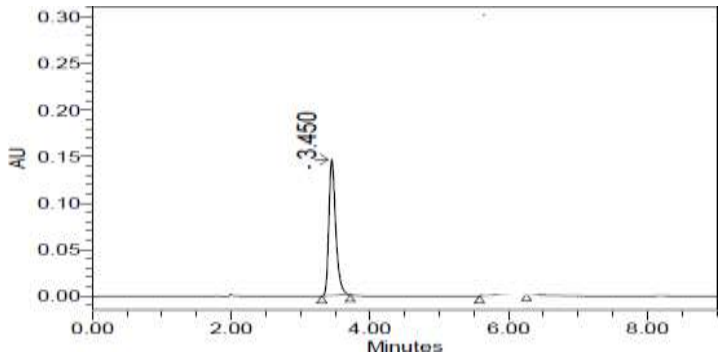


Figure 30: Method precision chromatogram 3

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Method precision 3	Cobimetinib	3.450	389776	3556	1.077

Table No. 13: Method precision data:

Sr. No.	RT	Std
1	4.573	567890
2	3.933	456732
3	3.450	389776
Average	3.985	471466
Std. Dev	0.0008	485.119
% RSD	0.05	0.13

7.4 ACCURACY

The accuracy for estimation of cobimetinib using methanol was determined by adding known amount of the analyte. The accuracy was calculated from the test results as the percentage of the analyte recovered by the assay.

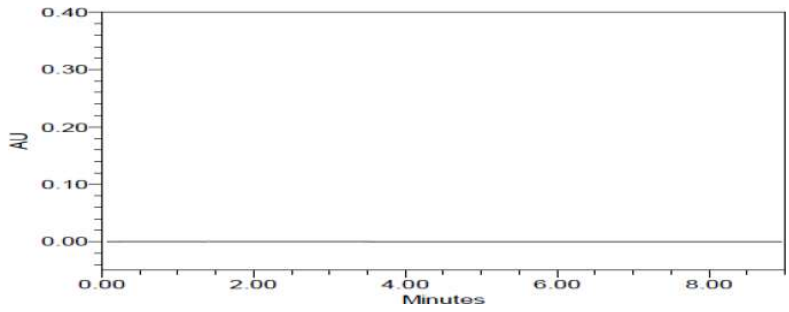


Figure 31: Chromatogram showing blank

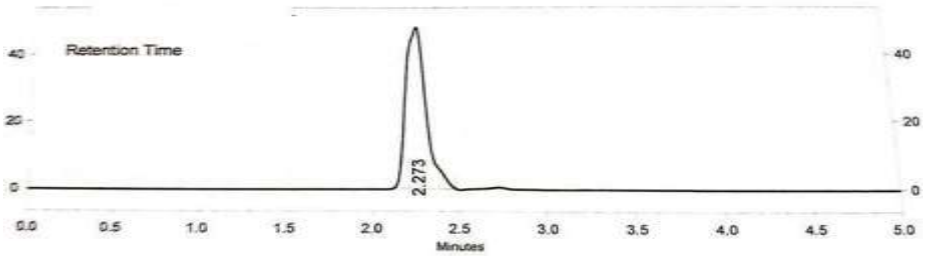


Figure 32: Standard Chromatogram

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Standard Accuracy	Cobimetinib	2.273	555447	3519	1.059

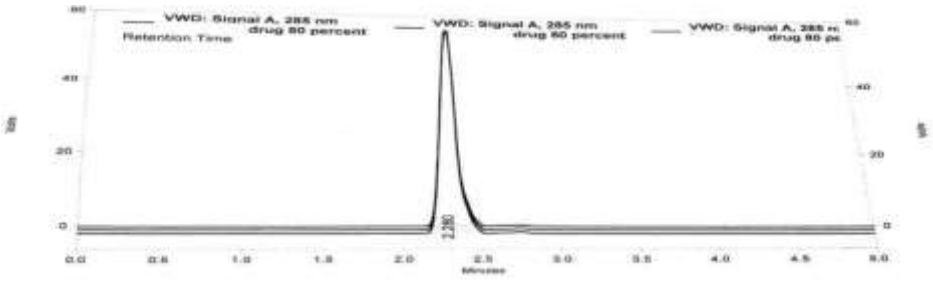


FIGURE NO. 33: Chromatography for 80 % Accuracy

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Accuracy 80%	Cobimetinb	2.280	6708805.667	3488	1.098

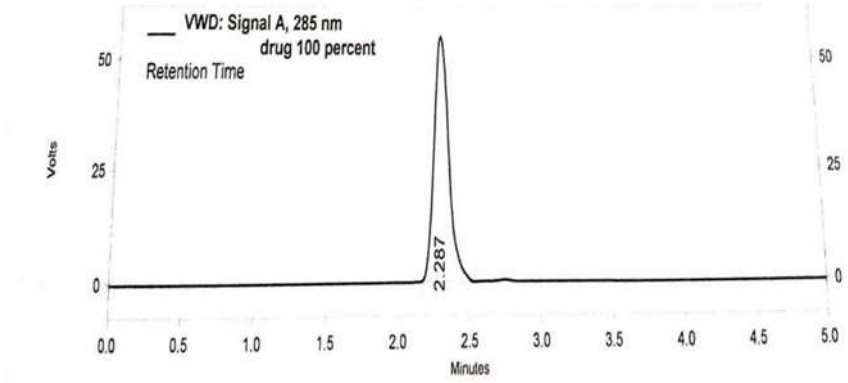


FIGURE NO. 34: Chromatography for 100 % Accuracy

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Accuracy 100%	Cobimetinib	2.287	6766496	3496	1.099

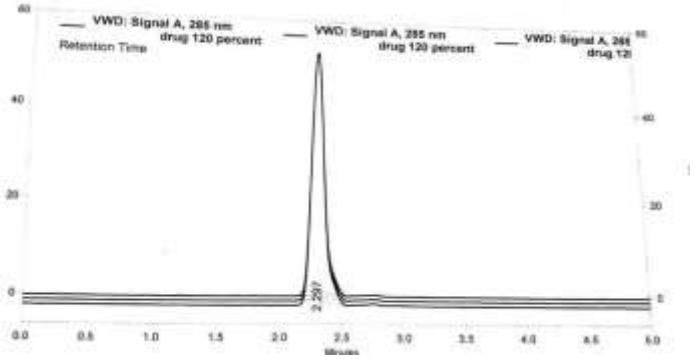


FIGURE NO. 35: Chromatography for 120 % Accuracy

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Accuracy 120%	Cobimetinib	2.297	6242954.33	34998	1.099

Table No. 14: Accuracy Standard Data

Name	Cobimetinib (cotellic)	
	RT	Area
Accuracy %80	2.280	6708805.667
Accuracy %100	2.287	6766496
Accuracy %120	2.297	6242954.33
Average	2.288	6572752

7.3 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ):

LOD and LOQ were calculated according to ICH recommendations where the approach is based on the signal-to-noise ratio. Chromatogram signals obtained with known low concentrations of analytes was compared with the signals of blank samples. A signal to noise ratio 3:1 and 10:1 was considered for calculating LOD and LOQ respectively.

Name of drug	LOD µg/ml	LOQ µg/ml
Cobimetinib	0.08	0.29

Table 15: LOD and LOQ for estimation of Cobimetinib

7.4 ROBUSTNESS

The robustness of an analytical procedures describes to its capability to remain unaffected by small and deliberate variation in the chromatographic conditions and found to be unaffected by small variation ±0.1ml/min in flow rate of mobile phase, and wavelength ±5nm result are shown.

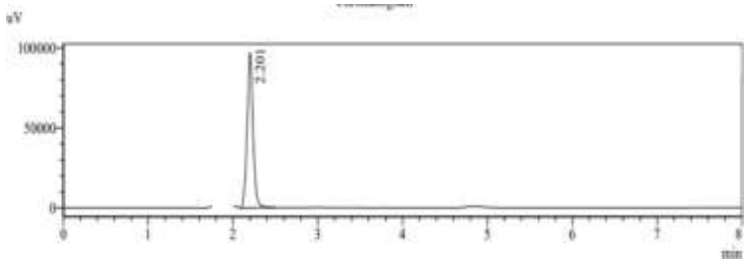


Figure 36: Chromatography for flow rate change 0.9 ml/minute for standard Observation:

Sr.No.	Name	RetentionTime	Area	Flow rate
1.	Cobimetinib	2.201	469067	0.90 ml

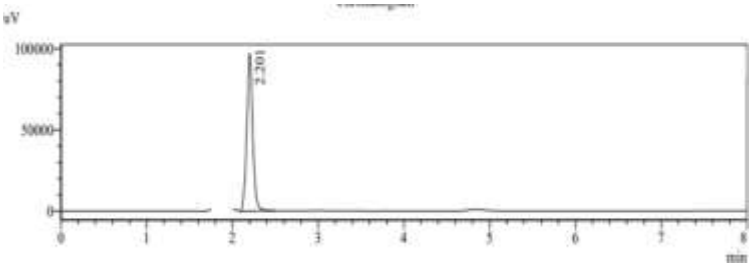


Figure 37: Chromatography for flow rate change 0.9 ml/minute for Sample Observation:

Sr.No.	Name	RetentionTime	Area	Flow rate
1.	Cobimetinib	2.201	644623	0.90 ml

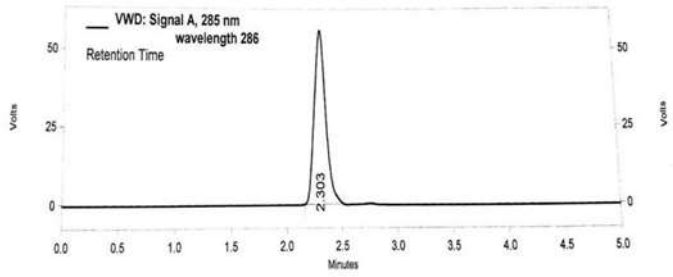


Figure 38:Chromatography for flow rate change 1.10 ml/minute for standard

Observation:

Sr.No.	Name	RetentionTime	Area	Flow rate
1.	Cobimetinib	2.303	387925	1.l0 ml

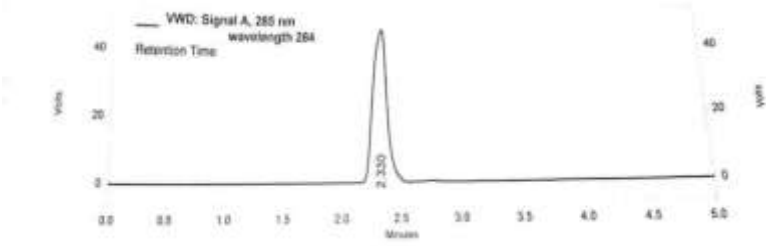


Figure 39: Chromatography for flow rate change 1.10 ml/minute for Sample Observation:

Sr.No.	Name	RetentionTime	Area	Flow rate
1.	Cobimetinib	2.330	534214	1.l0 ml

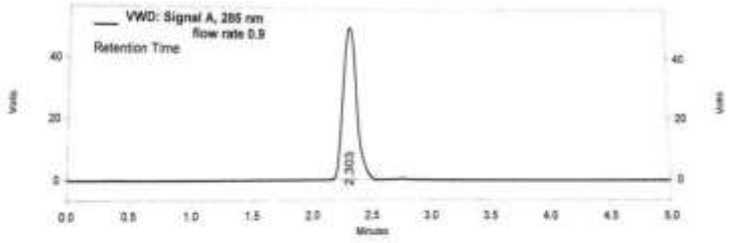


Figure 40: Chromatography for wavelength change 266 nm Standard

Observation:

Sr.No.	Name	RetentionTime	Area	Flow rate
1.	Cobimetinib	2.303	419948	266 nm

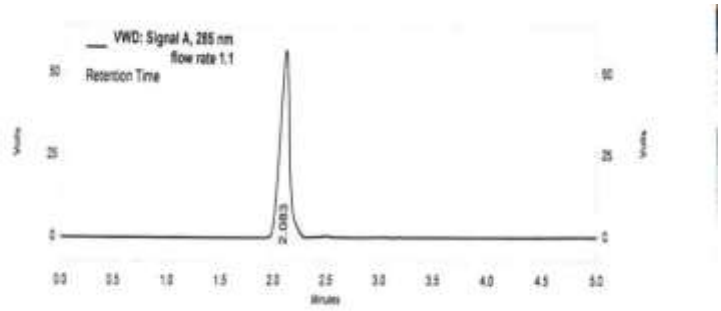


Figure 41: Chromatography for wavelength change 266 nm Sample

Observation:

Sr.No.	Name	RetentionTime	Area	Flow rate
1.	Cobimetinib	2.083	534214	266 nm

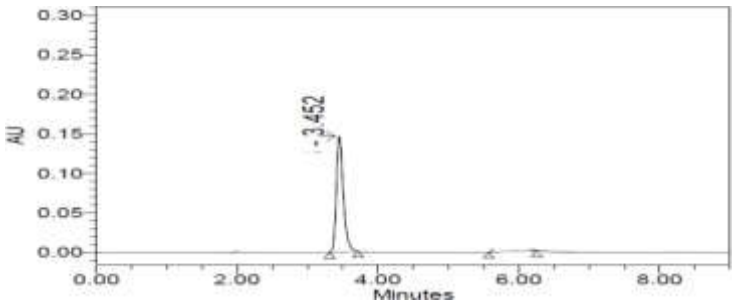


Figure 42: Chromatography for wavelength change 270 nm for Standard

Observation:

Sr.No.	Name	RetentionTime	Area	Flow rate
1.	Cobimetinib	3.452	391020	270 nm

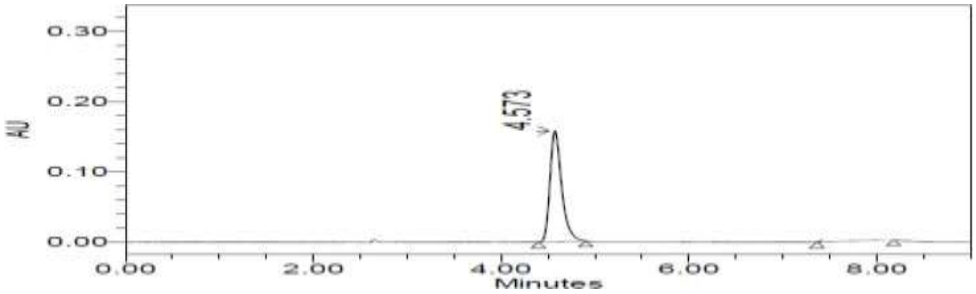


Figure 43: Chromatography for wavelength change 270 nm for Sample

Observation:

Sr.No.	Name	RetentionTime	Area	Flow rate
1.	Cobimetinib	4.573	535537	270 nm

Table No. 16: Effect of flowrate change and wavelength change

Flowrate Change : 0.9 ml/minute			
Std Area	**Spl Area**	**Assay Found**	**%**
469067	644623	5.04	100.9

Flowrate Change : 1.10 ml/minute				
	Std Area	**Spl Area**	**Assay Found**	**%**
	387925	534214	5.05	101.1
Wavelength Change: 266 nm				
	Std Area	**Spl Area**	**Assay Found**	**%**
	419948	575103	5.03	100.5
Wavelength Change: 270 nm				
	Std Area	**Spl Area**	**Assay Found**	**%**
	391020	534214	5.01	100.3

7.13 SPECIFICITY

Specificity is a procedure to detect quantitatively the analyte in presence of the components that may be expected to be present in the sample matrix. While selectivity is a procedure to detect the analyte qualitatively in presence of components that may be expected to be presented in the sample matrix. The excipients in tablet formulation were spiked in pre weighted quantity of drugs and then absorbance was measured and calculations were done to determine the quantity of the drug.

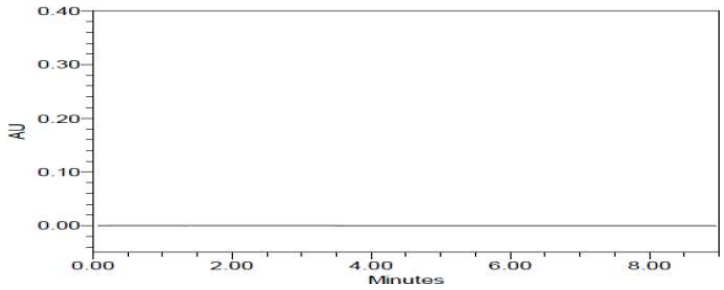


FIGURE NO. 44 Chromatogram for blank Chromatogram Specificity

7.7 Palacebo

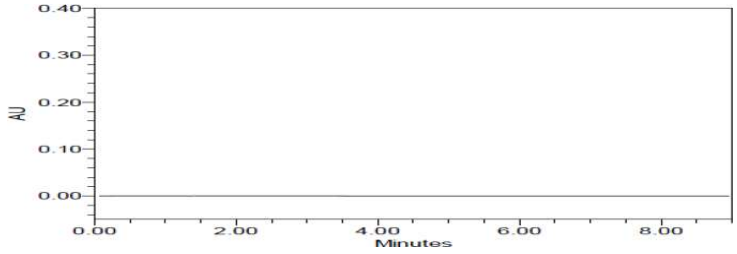


Figure 45: Placebo chromatogram

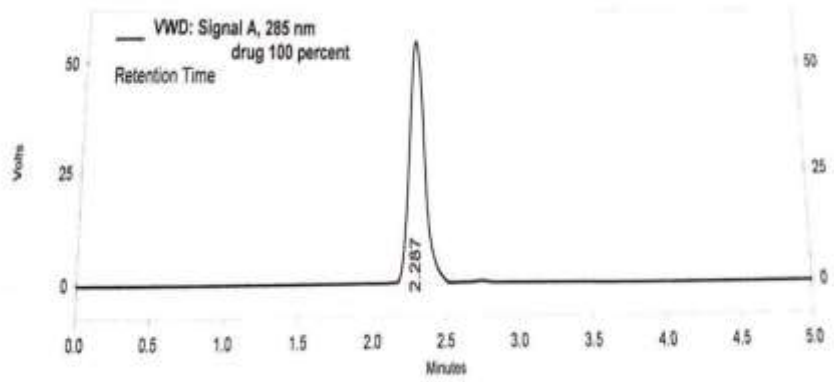


FIGURE NO. 46: Chromatogram for standard Chromatogram Specificity

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
specificity	Cobimetinib	2.283	23476	3499	1.898

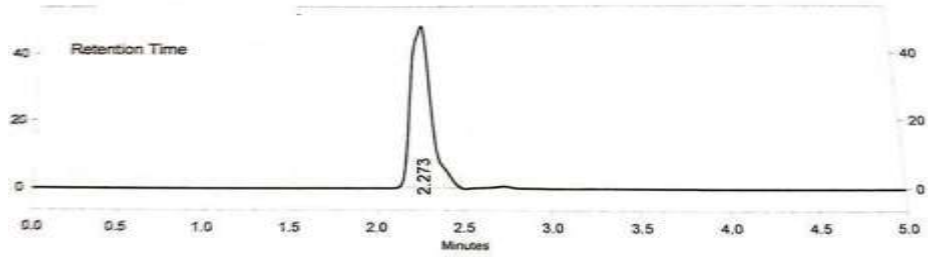


FIGURE NO. 47: Chromatogram for sample Chromatogram Specificity

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Specificity	Cobimetinib	2.273	23456	3496	1.899

Table No. 17: Observation for specificity

Sr. No.	Name	Cobimetinib	
		RT	Area
1.	**Blank**	-	-
2.	**Standard**	2.283	23476
3.	**Placebo**	-	-
4.	**Sample**	2.273	23456

7.7 Ruggedness:

Ruggedness is not addressed in the ICH documents (4,5) Its definition has been replaced by reproducibility, which has the same meaning as ruggedness, defined by the USP as the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

7.8.1 Ruggedness Day-1(Intra Day precision):

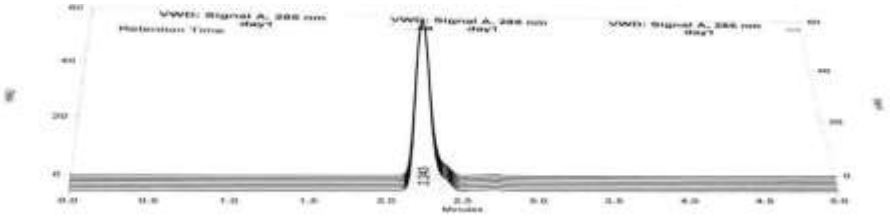


FIGURE NO. 48: Chromatogram showing day 1

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Ruggedness:	Cobimetinib	2.20	23456	3671	1.072

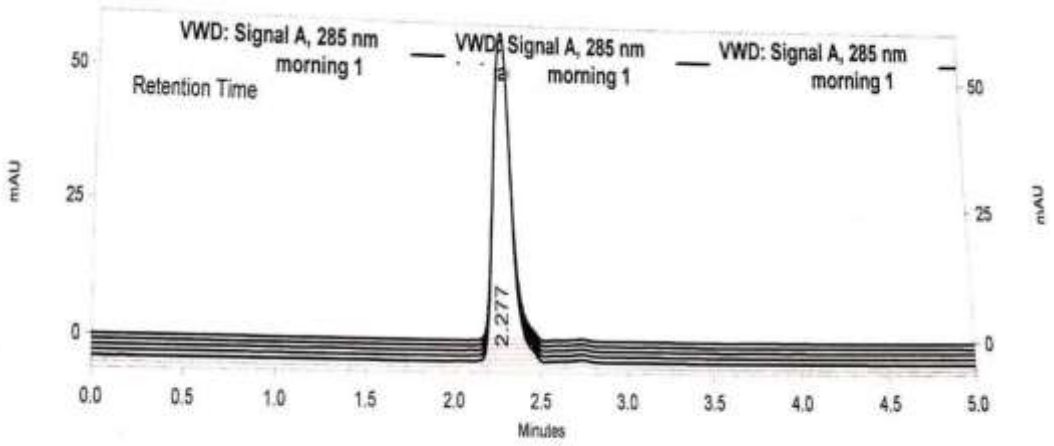


FIGURE NO. 49: Chromatogram showing intraday precision 2

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Ruggedness:	Cobimetinib	2.277	345678	3671	1.074

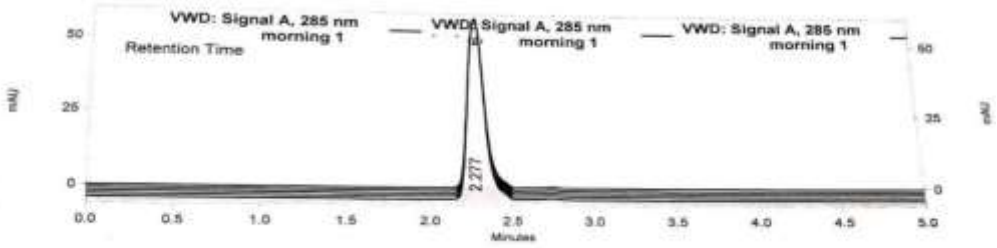


FIGURE NO. 50: Chromatogram showing inter precision 1

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Ruggedness:	cobimetinib	2.277	345378	3456	1.076

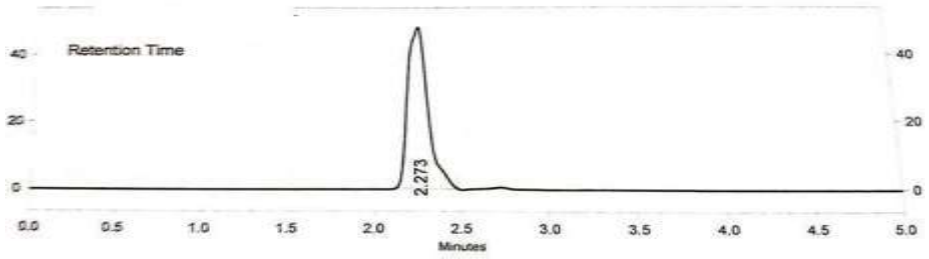


Figure 51: Chromatogram showing inter precision 2

Observation:

Title	Name	Ret Time	Area	USP Plate Count	Tailing Factor
Ruggedness:	Cobimetinib	2.273	345777	3452	1.45

Table No.18: Intra Precision Data

Sr	RT	Std Area
1	2.20	23456
2	2.277	345678
Avg	2.238	184567
Std dev	0.002	297.88
%RSD	0.11	0.07

Table No. 19: inter day Precision Data

Sr No	RT	Std Area
1	2.277	345378
2	2.273	345777
Avg	2.275	265072
Std dev	0.003	298.87
%RSD	0.12	0.08

8. SUMMARY

The method was developed and validated for system suitability, specificity, linearity, precision, accuracy, limit of detection and limit of quantification. The system suitability was found to be within the limits, the limit was not more than RSD‹2%. This indicates that the method is precise.

The data are all showed in the tables above. In view of the need for a suitable method for the quantification of related substances in Cobimetinib (cotallic), a newer, simple, accurate and low cost UHPLC method for the effective estimation and quantification of Impurities and the possible degradants in accelerated stability studies was successfully developed and validated. And also the present work is focused on quantification of Cobimetinib purity and to employee this method as the stability indicating method for related substances in Cobimetinib.

The forced degradation studies were performed for the developed method and this UHPLC method. was able to resolve the possible degradants. The proposed new analytical method was validated in accordance with ICH guidelines. The method is validated for the parameters like accuracy, linearity, precision, specificity, ruggedness, robustness and system suitability.

The detector response was found linear with a correlation co-efficient of 0.995 to 0.990 for all the known impurities in drug main peak. The developed method was found to be rapid, accurate, sensitive, and reproducible and could be used for routine analysis and quality control of pharmaceutical preparations containing related substances in Cobimetinib. Validation of the method was done in accordance with USFDA and ICH guidelines. The proposed method was found to be suitable for the routine pharmaceutical analysis in analytical laboratories.

9. CONCLUSION

In addition to positive requirements for analytical methods, the striking advantage of all the developed method is that they are economical, cheap, and precise. The proposed RP- HPLC methods were suitable technique for the determination of cobimetinib fumerate. All the parameters analyzing cobimetinib fumerate met the criteria of ICH guidelines for Method Validation. In the present investigation, we have developed a simple, sensitive,

precise and accurate RP- HPLC method for the quantitative estimation of cobimetinib fumerate in bulk and pharmaceutical formulations. The recoveries achieved were found good by the method. The HPLC method is more sensitive, precise and accurate compared to the spectrophotometric methods. The HPLC method developed may be recommended for the routine determination of cobimetinib fumerate in bulk drug and pharmaceutical formulations.

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