ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF BEMPEDOIC ACID AND EZETIMIDE IN COMBINE DOSAGE FORM BY RP-HPLC

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ABSTRACT:

Analytical Method Development and Validation for Bempedoic acid and Ezetimibe in bulk and Combined Dosage Form by RP-HPLC. Include RP-HPLC Method Development and Validation for Simultaneous Estimation of Bempedoic acid and Ezetimibe in Bulk and their Pharmaceutical dosage form. Using agilent tech. gradient system with auto injector HPLC system, equipped with an auto sampler injector with 10µl is injected eluted with the mobile phase containing methanol and acidic water (0.05%OPA) in the ratio of 75:25 v/v which is pumped at a flow rate of 1ml/min and detected by UV detector at 233nm. The peak of Bempedoic acid and Ezetimibe was eluted at retention times of 2.165 min and 5.099 min respectively. In this proposed HPLC method for the selected drugs showed good linearity. Results for the recoveries of selected drugs were found to be within limits (98 – 102 %). These indicate that the proposed method was accurate for the analysis.

Keywords: *Bempedoic acid, Ezetimibe, RP-HPLC.*

1. Introduction:

The science that deals with the identification and quantification of the components of material systems is called analytical science. It is so called because the process of determining the level of any or all components in a material system is called analysis. It involves both physical and chemical processes. The chemical processes, it is called chemical analysis or more broadly, analytical chemistry.1

Analytical chemistry deals with methods for determining the chemical composition of samples of matter. A qualitative method yields information about the identity of atomic or molecular

species or the functional groups in the samples; a quantitative methods, in contrast provides numerical information as to the relative amount of one or more of these components.2

Modern analytical chemistry generally requires precise analytical measurements at very low concentrations, with a variety of instruments. Frequently, high-resolution separations have to be achieved with selective chromatographic methods prior to analytical determinations. Therefore, the knowledge of instrumentation used in chemical analysis today is of paramount importance to assure future progress in various fields of scientific endeavor. This includes various disciplines of chemistry such as pharmaceutical chemistry, medicinal chemistry, biochemistry, biotechnology, and environmental sciences.3

In summary, a more appropriate description of analytical chemistry is "the science of inventing and applying the concepts, principles, and strategies for measuring the characteristics of chemical systems and species."4

Analytical method development is the heart of analytical chemistry. It involves development and validation of new analytical method for the purpose of testing samples. Quality control samples are tested using UV, IR, HPLC, GC, LC-MS and HPTLC etc.5-7

The stages or steps in an overall analytical procedure can be summarized as follows.
- Definition of the problem
- Choice of technique and method
- Preparation of sample
- Qualitative analysis
- Quantitative analysis
- Calculating the results and presenting the data.
- Review of the original problem

1.1. Types of Analytical Techniques

1.1.1. Spectroscopic Techniques

- UV-Visible Spectroscopy
- Infra-Red Spectroscopy
- Raman Spectroscopy
- Atomic Absorption Spectroscopy
- X-Ray Diffraction Spectroscopy
- Mass Spectroscopy
- NMR Spectroscopy

1.1.2. Chromatographic Techniques

- Gas Chromatography
- Liquid Chromatography

- Size Exclusion Chromatography
- High Performance Thin Layer Chromatography
- Paper Chromatography

1.1.3. Electrochemical Techniques

- Potentiometry
- Voltammetry
- Amperometry
- Coulometry
- Electrogravimetry
- Conductometry

CHROMATOGRAPHIC TECHNIQUES:

Chromatography is the most frequently used analytical technique in pharmaceutical analysis for the separation, identification and determination of the chemical components in complex mixtures. No other separation method is as powerful and generally applicable as chromatography. An understanding of the parameters which governs chromatographic performance has given rise to improvements in chromatography systems, so the ability to achieve high resolution separations is continually increasing.8-9

a) Normal-phase chromatography:

The term "normal phase" is used to denote a chromatographic system in which a polar stationary phase is employed and a less polar mobile phase is used for elution of the analyte. In the normal phase mode, neutral solutes in solution are separated on the basis of their polarity; the more polar the solute, the greater is its retention on the column. Since the mobile phase is less polar than the stationary phase, increasing the polarity of the mobile phase results in decreased solute retention.10-11

b) Reversed phase chromatography:

Reversed-phase chromatography, the most widely used chromatographic mode, is used to separate neutral molecules in solution on the basis of their hydrophobicity. As the name indicates, reversed phase chromatography is reverse of normal phase chromatography in the sense that it involves the use of a non-polar stationary phase and a polar mobile phase. As a result, a decrease in the polarity of the mobile phase results in decrease in solute retention.12-13

High Performance Liquid Chromatography:

A form of column chromatography commonly used in analytical chemistry to separate, identify and quantify small molecules, which utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on

the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. Schematic of an apparatus for HPLC is given in Figure 1.14

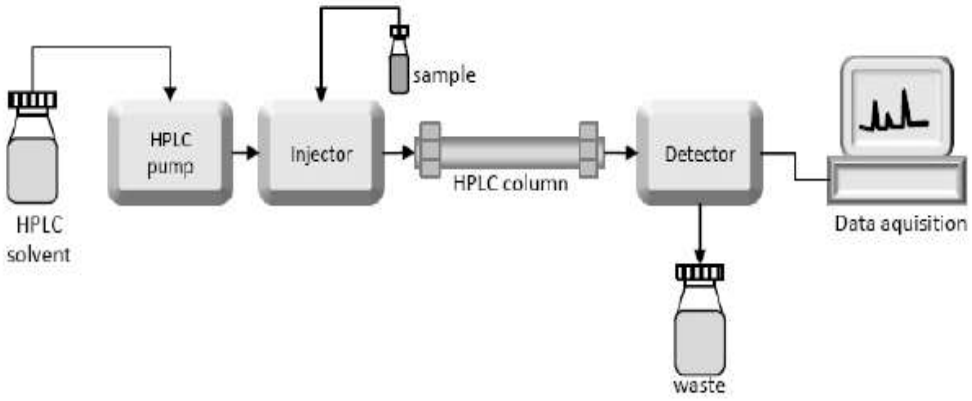


Figure: 1 Schematic of an apparatus for HPLC

A. Mobile phase reservoirs and solvent treatment system:

The reservoirs may be made up of glass or stainless steel containing up to 500 ml of solvent. They are also provided with means for removing dissolved gases like oxygen and nitrogen, which may bubbles in the column and the detector system. Degassing: Many liquids dissolves appreciable amounts of atmospheric gases e.g. air, suspended air bubbles may be a major cause of practical problems in HPLC, especially affecting the operation of pump and detector. However, all such problems may be avoided by degassing the mobile phase under vacuum or distillation, sparging with fine spray of an inert gas of low solubility such as argon or helium or by heating and ultrasonic stirring.

B. Pumps:

The pump is one of the most important components of the HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity. Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10000 psi or at least 6000 psi. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical proposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rates as most HPLC detectors are flow sensitive and errors in quantization will result from changes in flow rate. These pumps are necessary to force the liquid (mobile phase) through the column with finely packed particles. The pumps are categorized as,

Constant displacement pump:

It is a low pulsation displacement pump comprises two series - connected cylinders, which are controlled by valves so that the delivery takes place only in one direction. The pistons of the cylinders are controlled through cams which are driven jointly at a constant velocity.

Reciprocating pump:

The piston expels liquid through a one-way valve (check valve). The pumping rate is usually adjusted by controlling the distance the piston retracts, thus limiting the amount of liquid pushed out by each stroke, or by the cam rotating speed (Figure 2).

C. Sample injection system:

A variety of sample introduction devices exists, manual and automatic as shown in following Figure 3

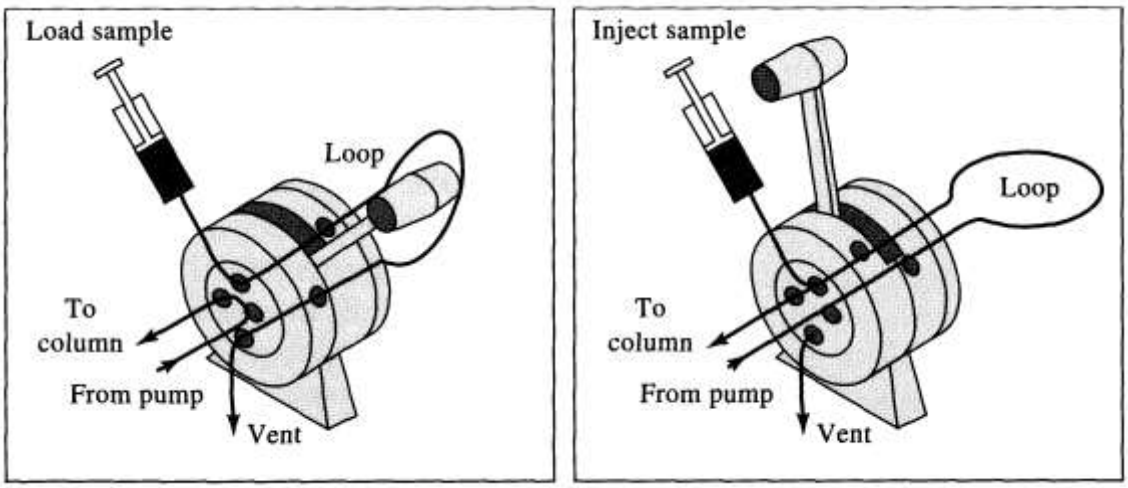


Figure: 3 Sample loading and injection system

D. **Columns**:

Column plays a key part of the HPLC system which is made up of stainless steel material (Highly polished surface) with external diameter (6.35mm or 0.25 inch), internal diameter (4-5mm - usually: 4.6 mm) and length: 10-30 cm (usually: 25 cm). Each end of the column is adequately fitted with stainless steel gauze or fit with a mesh of 2 µm or less so as to retain the packing material (usually having a particle diameter 10, 5 and 3µm) and they are called as fittings. The unions are used which connects the column and detectors. HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet

connection to keep dispersion to a minimum. Some state of the art systems are now 'chip' based and may use no particles at all. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase.15

Guard columns:

Usually, a short guard column is introduced before the analytical column to increase the efficiency of the analytical column by removing not only particulate matter and contaminants from the solvents but also sample components that bind irreversibly to the stationary phase. The composition of the guard column packing should be closely similar to that of analytical column; the particle size is usually larger to minimize pressure drop.

E. Detectors:

The important role of the HPLC detector is to monitor the solutes as they are eluted from the column. The detector generates an electrical signal that is proportional to the level of some property of mobile phase or solutes.

Quantitative analysis in HPLC:

The basic theory for quantification involves the measurement of peak height or peak area. To determine the concentration of a compound, the peak area of standard against the sample is checked. The various calibration methods are as follows.

a. External Standard Calibration: This is the simplest method amongst all. The accuracy of this method is dependent on the reproducibility of the injection of sample volume. To perform this method, a standard solution of known concentration of the compound of interest is prepared. A fixed amount, which should be similar in concentration to the unknown, is injected. Peak height or area is plotted versus the concentration for each compound .

b. Internal Standard Calibration: This method tends to most accurate and precise results. In this method, equal amount of internal standard, a component that is not present in the sample, is added to both the sample and standard solution. Quantification is achieved by using ratios of peak height or area of the component to the internal standard. The concentration of unknown =

c. Standard addition method: This is especially useful when there is problem with interference from sample matrix, since it cancels out these effects. To perform this quantification, the sample is divided into two portions so that a known amount of analyte (spike) can be added to one portion. These two samples, the original and along with spike, are then analysed. The sample with the spike shows a larger analytical response than the original

sample because of the additional amount of analyte. The method has a drawback if only a small volume of sample is available.

Steps involved in HPLC Method Development:

1. Information on sample, define separation goals
2. Need for special HPLC procedure, sample pretreatment
3. Choose detector and detector settings.
4. Choose LC method, preliminary run
5. Optimize separation conditions
6. Check for Problems
7. Recovery of purified material
8. Quantitative calibration
9. Qualitative method.
10. Validate the method

Validation of analytical method:

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics, which need to be evaluated. Typical validation characteristics that should be considered are as follows, 16

1. Analytical procedure: The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. Reference standard and the reagents preparation, use of apparatus, generation of calibration curve and use of the formulae for the calculation, etc.17

2. Accuracy: The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. In the case of the assay of a drug substance, accuracy may be determined by application of the analytical method to an analyte of known purity (e.g., a reference standard). It is often expressed as % recovery by analyzing known added amounts of analyte. Also, it can be determined by applying the procedure to quantitatively prepared samples. Accuracy is calculated as the percentage recovery by the assay of the known added amount of analyte in the pre analyzed sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

3. Precision: The precision of analytical procedure expresses closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions. It may be considered at three levels: repeatability, intermediate precision and reproducibility**.** It is expressed as standard deviation or coefficient of variation. The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate standard deviation or relative standard deviation.18

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

Intermediate precision: Intermediate precision expresses within-laboratories variations, different days, different analyst, different equipment, etc.

Reproducibility: Reproducibility expresses the precision between laboratories.

4. Limit of Detection (LOD): The Limit of detection (LOD) of an individual analytical procedure is the lowest amount of an analyte that can be detected in given sample, which can be detected but not necessarily quantitated under the stated experimental conditions as an exact value. The detection limits is usually expressed as the concentration of analyte (e.g., percentage, ppb) in the sample. The detection limit can be determined based on signal to noise ratio which is 3:1.19-21

Based on visual evaluation: The detection limit 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.

Based on signal to noise ratio: A signal to noise ratio between 3:1 is generally considered acceptable for estimating the detection limit.
Based on standard deviation of the response and slope: The limit of detection (LOD) may be expressed as,

$$LOD = \frac{3.3\,\sigma}{S}$$

Where,
σ = the standard deviation of the response.
S = slope of calibration curve of analyte.

5. Limit of Quantitation (LOQ): The quantitation limit of an individual analytical procedure is the lowest amount of analyte that can be detected quantitatively from sample, with suitable acceptable precision and accuracy under the stated experimental conditions. It is expressed as the concentration of analyte (e.g., percentage, ppm) in the sample. Some approaches listed below may be acceptable.

Based on visual evaluation: The quantitation limit is determined by the analysis of samples with known concentrations of analyte.

Based on signal to noise ratio: Signal to noise ratio 10:1 is generally considered acceptable.

Based on standard deviation of the response and slope: The quantitation limit can be determine based on signal to noise ratio in which ratio 10:1 is acceptable and based on standard deviation method. The limit of quantitation (LOQ) may be expressed as

$$LOQ = \frac{10\,\sigma}{S}$$

Where,
σ = the standard deviation of the response.
S = slope of calibration curve of analyte

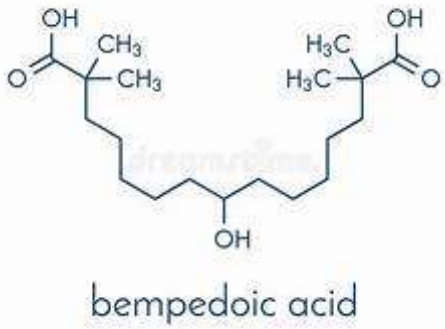
6. Linearity: The linearity of an analytical procedure is the ability to obtain test results, which are directly proportional to the concentration of an analyte in the sample within a given range. It should be established across the range of the analytical procedure.

7. Range: The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure is of precision, accuracy and linearity can be established using the method. The range is normally expressed in the same unit as test result.

8. Robustness: The robustness of 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. on studies.22-24

2. Drug Profile

Table no.2 DRUG PROFILE OF BEMPEDOIC ACID: -

Particular	Bempedoic Acid
Category	antibiotic
Structure	 bempedoic acid
IUPAC Name	8-Hydroxy-2,2,14,14-tetramethylpentadecanedioic acid
Molecular formula	$C_{19}H_{36}O_5$
Molecular weight	344.492 g·mol^{-1}
CAS No.	738606-46-7
Description	Crystalline, white to off white powder

Melting point	87-92 °C
Pka	5.35
Solubility	Soluble in organic solvent such as ethanol, dimethyl formamide and sparingly soluble in aqueous buffers.
Protein Binding	97 to 99%
Half life	15-24 hr.

Mechanism of Action:

Bempedoic acid is a prodrug. It is activated to the thioester with coenzyme A by the enzyme SLC27A2 in the liver. The activated substance inhibits ATP citrate lyase, which is involved in the liver's biosynthesis of cholesterol upstream of HMG-CoA reductase, the enzyme that is blocked by statins.

The substance also activates AMP-activated protein kinase, but this effect is likely not relevant in humans.

Pharmacokinetics:

Absorption

Bempedoic acid is rapidly absorbed in the small intestine. The Tmax of the 180mg tablet is estimated at 3.5 hours

Route of Elimination

Bempedoic acid's conjugates are primarily eliminated via the urine (70%) and the feces (30%). A total of 5% of the unchanged drug is excreted in the urine and feces, combined.

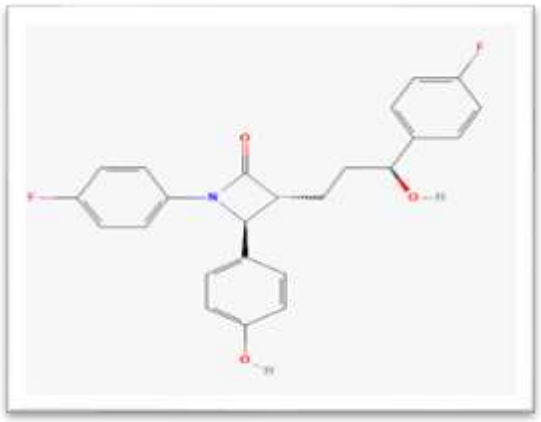
Metabolism

The two main metabolites of bempedoic metabolism are ETC-1002-CoA and ESP15228. Bempedoic acid is primarily eliminated via the metabolism of its acyl glucuronide. This drug is reversibly converted to an active metabolite (ESP15228) based on observations during in vitro studies. Both compounds resulting from the metabolism of bempedoic acid are metabolized to become inactive glucuronide conjugates by the enzyme UGT2B7.

DRUG PROFILE ON EZETIMIBE :-

Table no.3 Drug Profile OF Ezetimibe

Particular	Ezetimibe
Category	Anticholesteremic Agents

Structure	
IUPAC Name	(3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidin-2-one
Molecular formula	$C_{24}H_{21}F_2NO_3$
Molecular weight	409.4 g/mol
CAS No.	163222-33-1
Description	White, solid powder.
Melting point	163°C
Pka	4.5
Solubility	Insoluble in water; soluble in ethanol, methanol, acetone, DMSO, DMF.
Protein Binding	98% to 99% (in vitro concentration of 5 to 500ng/mL)

MechanismofAction

Ezetimibe mediates its blood cholesterol-lowering effect via selectively inhibiting the absorption of cholesterol and phytosterol by the small intestine without altering the absorption of fat-soluble vitamins and nutrients. The primary target of ezetimibe is the cholesterol transport protein Niemann-Pick C1-Like 1 (NPC1L1) protein. NPC1L1 is expressed on enterocytes/gut lumen (apical) as well as the hepatobiliary (canalicular) interface and plays a role in facilitating internalization of free cholesterol into the enterocyte in conjunction with the adaptor protein 2 (AP2) complex and clathrin. Once cholesterol in the gut lumen or bile is incorporated into the cell membrane of enterocytes, it binds to the sterol-sensing domain of NPC1L1 and forms a NPC1L1/cholesterol complex. The complex is then internalized or endocytosed by joining to AP2 clathrin, forming a vesicle complex that is translocated for storage in the endocytic recycling compartment.

Pharmacokinetics

Absorption: Administration of a single 10-mg dose of ezetimibe in fasted adults resulted in peak plasma concentrations (C_{max}) of 3.4-5.5 ng/mL within 4-12 hours (T_{max}). The C_{max} of the major pharmacologically-active metabolite, ezetimibe-glucuronide, was 45-71 ng/mL and its T_{max} was 1-2 hours. Food consumption has minimal effect on ezetimibe absorption, but the C_{max} is increased by 38% when administered alongside a high-fat meal. The true bioavailability of ezetimibe cannot be determined, as it is insoluble in aqueous media suitable for intravenous injection.

Route of Elimination

78% and 11% of orally administered radiolabelled ezetimibe are recovered in the feces and urine, respectively. Unchanged parent drug is the major component in feces and accounts for approximately 69% of an administered dose, while ezetimibe-glucuronide is the major component in urine and accounts for approximately 9% of an administered dose. High recovery of unchanged parent drug in feces suggests low absorption and/or hydrolysis of ezetimibe-glucuronide secreted in the bile.

Volume of Distribution

The relative volume of distribution of ezetimibe is 107.5L.

Metabolism

Ezetimibe is rapidly and extensively metabolized via a phase II glucuronide conjugation reaction in the small intestine and liver to form its main phenolic metabolite, ezetimibe glucuronide. The main human liver and/or intestinal uridine 5′-diphosphate (UDP)-glucuronosyltransferase (UGT) enzymes responsible for the glucuronidation of ezetimibe were shown to be UGT1A1, 1A3, and 2B15 _in vitro_. Minimal phase I reaction involving oxidation of ezetimibe also occurs to form SCH 57871, and human jejunum microsomes also produced trace levels of a benzylic glucuronide.

Biological Half- Life

Both ezetimibe and ezetimibe-glucuronide display an approximate half-life of 22 hours

Side effect:

- Cold symptoms such as stuffy nose, sneezing, sore throat (3.7%)
- Muscle pain (3.2%)
- Upper respiratory tract infection (2.9%)
- Joint pain (2.6%)
- Diarrhea

3.MATERIAL AND INSTRUMENTS

3.1 Selection and Procurement of Drug

Drug sample supplier

Table 4: Drug and Drug Supplier

Name of Drug	Drug Supplier
Bempedoic acid and Ezetimibe	Swapnroop drug and pharmaceutical

List of reagents & chemicals used

Table 5: List of Reagents and Chemicals used

Sr. No.	Name of chemicals	Manufacturer.
1.	Methanol (HPLC grade)	Merck Ltd., India
2.	Acetonitrile (HPLC grade)	Merck Ltd., India
3.	Potassium phosphate buffer (HPLC grade)	Merck Ltd., India

3.2 Selection of formulation:

Marketed Preparation**:**

The marketed preparation was obtained from local market and is referred here after in this thesis by the name as such.

Brand Name: Bempetol EZ
Content: Bempedoic acid and Ezetimibe
Marketed by: Castor Lifecare Pvt. Ltd**.**

3.3 Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Bempedoic acid and Ezetimibe.

Instruments:

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, (DAD) & Gradient Detector. Equipped with Reverse Phase (Agilent) C_{18} column (4.6mm x 250mm; 5µm), and UV730D Absorbance detector and running chemstation 10.1 software.

3.4 Instruments and Equipments

Table. 6: Instrument (HPLC) Details used during Method Development

	Name of Instrument	Company Name
1	HPLC Instrument	Agilent Tech. Gradient System with Auto injector (Chemstation software)
2	UV-Spectrophotometer	Analytical Technology
3	Column(C_{18})	Agilent C_{18} (250mmX 4.6mm,5µm) :
4	pH meter	VSI pH meter(VSI 1-B)
5	Balance	WENSAR™ High Resolution Balance
6	Sonicator	Ultrasonics electronic instrument

4. EXPERIMENTAL WORK:

4.1 HPLC:

4.1.1 Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Bempedoic acid and Ezetimibe.

Instruments:

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector,DAD Detector. Equiped with Reverse Phase C_{18} (Agilent) with 250mm x4.6; (5µm), UV730D Absorbance detector and running chemstation 10.1 software.

Table 7: List of instruments

	Name of Instrument	Company Name
1	HPLC Instrument	Agilent Tech. Gradient System with Auto injector
2	UV-Spectrophotometer	Analytical Technologies Limited
3	Column(C_{18})	Agilent C_{18} (250mmX 4.6mm,5µm)
4	pH meter	VSI pH meter(VSI 1-B)
5	Balance	WENSAR™ High Resolution Balance.

6	Sonicator	Ultrasonics electronic instrument

a) Chromatographic conditions:

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

Table No.8: chromatographic conditions (HPLC) details used during method Development

1.	HPLC	Agilent Tech. Gradient System with Auto injector
2.	Software	Chemstation
3.	Column	(Agilent) C18 column (4.6mm x 250mm)
4.	Particle size packing	5 μm
5.	Stationary phase	C-18 (Agilent)
6.	Mobile Phase	Methanol: Water(0.05% OPA) 75 : 25
7.	Detection Wavelength	233 nm
8.	Flow rate	1 ml/min
9.	Temperature	Ambient
10.	Sample size	20 μl
11.	pH	3
12.	Run Time	10 min
13.	Filter paper	0.45 μm

4.2 Study of Bempedoic acid and Ezetimibe on the chromatographic conditions used in method development of HPLC for the Following Mobile phase were tried:

4.2.1 METHOD DEVELOPMENT OF HPLC:

- **List of Mobile Phase :**
- **Table No.9: Selection of mobile Phase.**

Sr.No.	Mobile Phase
1.	Methanol : water (0.05%OPa PH-3)(80:20 % v/v)
2	Methanol : Water 0.05 OPA (70 : 30%v/v)PH3
3	Methanol : Water 0.05% OPA(50 : 50%v/v)PH3
4	Methanol : Water 0.05%OPA (90 : 10%v/v)PH3
5	Methanol : Water 0.05%OPA (75 : 25%v/v)PH3

4.3. Analysis of standard drugs was done by following parameters:

- Melting point
- Solubility
- UV spectra and λ_{max}
- HPLC chromatogram and retention time

4.4. Selection of wavelength by UV-Visible Spectrophotometry:-

4.5.1. Preparation of standard stock solution:-

- **Bempedoic acid standard stock solution : (Stock I)**

An accurately weighed quantity, 180 mg of Bempedoic acid (BPD) was dissolved in Methanol in a 50 ml volumetric flask and volume made up to 50.0 ml to produce a solution of 3600 µg/ml.

- **Ezetimibe standard stock solution : (Stock II)**

An accurately weighed quantity, 10 mg of Ezetimibe (ETM) was dissolved in Methanol in 50 ml volumetric flask and volume made up to 50.0 ml to produce a solution of 200 µg/ml.

- **Preparation of Stock Standard Combination Solution :(Stock III) [FSD+MTS]**

Accurately weight and transfer 180 mg Bempedoic acid and Ezetimibe 10 mg working standard into 50 ml volumetric flask as about diluent Methanol completely and make volume up to the mark with the same solvent to get 3600 µg/ml standard BPD and 200 µg/ml for ETM (stock solution) and 15 min sonicate to dissolve it and remove the unwanted gas, further an aliquots portion of Bempedoic acid and Ezetimibe stock solution in ratio of 75:25% were mixed in volumetric flask in 10 ml and volume was adjusted up to mark with mobile phase from the resulting solution 0.2 ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with Methanol: Acidic water(0.05% OPA) , prepared in (7.5ml Methanol: 2.5 ml OPA)solvent .Result as shown as; **(Fig No:14)**

4.5.2. HPLC used for chromatographic condition apply on the Preparation of standard solution:-

- **Preparation of std. Bempedoic acidsolution: (Stock I)**

From the freshly prepared standard stock solution (3600 µg/ml), 0.2 ml stock solution was pipeted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration of 72 µg/ml.

- **Preparation of std. Ezetimibe solution: (Stock II)**

From the freshly prepared standard stock solution (200 µg/ml), 0.2 ml stock solution was pipeted out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 4 µg/ml.

- **Preparation of std. Bempedoic acid and Ezetimibe solution :(Stock III)**

From the freshly prepared standard stock solution (3600+200 µg/ml), 0.1 ml stock solution was pipette out in 10 ml of volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 36+2 µg/ml.

4.5.3. Selection of mobile phase:

Each mobile phase was vacuum degassed and filtered through 0.45µ membrane filter. The mobile phase was allowed to equilibrate until steady baseline was obtained. The standard solution containing mixture of Bempedoic acid and Ezetimibe was run with different individual solvents as well as combinations of solvents were tried to get a good separation and stable peak. From the various mobile phases tried, mobile phase containing Methanol & Acidic water with pH adjust (3)OPA was selected since it gave sharp, well resolved peaks with symmetry within the limits and significant reproducible retention time for Bempedoic acid and Ezetimibe. Chromatograms of Bempedoic acid and Ezetimibe are shown in **(Table No: 12)** respectively.

4.6. Studies of Calibration plot :-

4.6.1. Optimization of Chromatographic condition :

The following chromatographic conditions were established by trial and error and were kept constant throughout the analysis.

Column	: C18 (250 mm× 4.6mm)
Particle size packing	: 5µm
Detection wavelength	: 233 nm
Flow rate	: 1 ml/min
Temperature	: Ambient
Sample size	: 20 µl
Mobile phase	: Methanol: water (0.05%OPA) (75 : 25)

4.7. Procedure for calibration curve of Bempedoic acidand Ezetimibe:

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. From the freshly prepared standard stock solution, pipette out 180 mg Bempedoic

acid and 10 mg Ezetimibe in 50 ml of volumetric flask and diluted with mobile phase. From it 0.1, 0.2, 0.3, 0.4 and 0.5ml of solution were pipette out in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 36,72,108,144,180 µg/ml of Bempedoic acid and 2,4,6,8,10 µg/ml of Ezetimibe. sample were injected and peaks were recorded at 233 nm as the graph plotted as concentration of drug verses peak area is depicted in **(fig. no. 15, 16)** respectively.

4.8. Study of system suitability parameters:[25]

The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from five replicate injections of standard solution.

4.9.1. Calibration Experiment:

- **RP-HPLC Method :**

a) Preparation of Calibration curve standard:

The above standard stock solution (3600:200µg/ml) of Bempedoic acidand Ezetimibe was diluted with mobile phase to yield Five calibration curve (cc) standards with concentrations of 36,72,108,144,180 µg/ml of Bempedoic acid and 2,4,6,8,10 µg/ml of Ezetimibe **(Table No.14) & (Table No.15).**

b) Calibration standard drug and regression equation data :

From the standard stock solution of Bempedoic acidand Ezetimibe, different concentration were prepared respectively in the range of 36-180 µg/ml for Bempedoic acid **(Figure No:15)** and 2-10 µg/ml for Ezetimibe and measured at 236 nm and 227 nm. The calibration curves were plotted **(Figure No:15)** and Regression equation data presented in **(Table No: 14 and Table No: 16).**

c) Calibration runs and regression analysis :

These calibration standard solutions were analyzed in three replicates using the under mentioned chromatographic conditions.

- Analytical column : Agilent C18 Column (250mm x 4.6mm, 5µm partical size).
- Injection volume : 20µl.
- Flow rate : 1 ml/min.
- Mobile phase : Methanol: Acidic water(0.05%0PA) (75: 25 % V/V).
- Detection : 233 nm.

4.10. Validation of method for analysis of Bempedoic acid and Ezetimibe:[26]

- The developed method was validated as per ICH guidelines.

4.10.1 Linearity:

Linearity of an analytical method is its ability to elicit test results that are directly or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range, The Result are shown in; **(Table No 17,19).**

- **Determination:**
  The linearity of the analytical method is determined by mathematical treatment of test results obtained by analysis of samples with analyst concentrations across the claimed range. Area is plotted graphically as a function of analyst concentration. **(Fig No. 17)** Percentage curve fittings are calculated**.** The Result are shown in; **(Table No.16);(Fig No.18 and Fig No. 27).**
- **Acceptance Criteria:**
  The plot should be linear passing through the origin.
  Correlation Coefficient should not be less than 0.999. The Result are shown in;
- **Preparation of standard stock solution for linearity:**
  Weight of drug (180 mg of Bempedoic acid and 10mg of Ezetimibe) were weighed and transfered to 50 ml volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. 0.05 ml of this solution diluted upto 10 ml volumetric flask with diluents was added to make up the volume.
- **Preparation of linearity solution:**
  A series of standard preparations of working standard of were prepared.

Table No.10:Table of linearity for Rp-HPLC Method

Concentration (µg/mL)	
Bempedoic acid	Ezetimibe
36	2
72	4
108	6
144	8
180	10

4.10.2 Accuracy (recovery):

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyst. The accuracy of an analytical method is determined by applying the

method to analyzed samples, to which known amounts of analyst have been added. The accuracy is calculated from the test results as the percentage of analyst recovered by the assay, The RP-HPLC Method Result are shown in; **(Table No:20,21).**

- **Acceptance Criteria:**
  Mean recovery should be in the range of 98-102%.
  The Relative Standard Deviation should not be more than 2.0%.
- **Preparation of standard stock solution:**
  180 mg of Bempedoic acid and 10 mg of Ezetimibe working standards were weighed and transferred to 50 ml volumetric flask & diluent was added to make up the volume 0.1 ml of this solution diluted upto 10 ml with diluent.
- **Application of proposed method for analysis of tablet formulation:**
- **Accuracy**

The accuracy was determined by Bempedoic acid and Ezetimibe (equivalent to 180 mg of Bempedoic acid and 10 mg of Ezetimibe (80 %, 100 % and 120 % of the label claimed, respectively) to quantity equivalent to average weight of marketed tablets. This powder mixture containing 180 mg of Bempedoic acid and 10 mg of Ezetimibe were triturated and then subjected to chromatographic analysis using the described method. The resulting mixtures were analyzed in duplicates over a day. The % recovery of added drug was taken as a measure of accuracy**.**
The Result are shown in; **(Fig No : 30,31,32)**

Table No. 11 :Table of Accuracy Rp-HPLC Method

Sample	**Amount Added (mg)**	
	Bempedoic acid	Ezetimibe
Accuracy 80%	28.8	1.6
Accuracy 100%	36	2
Accuracy 120%	43.2	2.4

4.10.3 Repeatability:

Precision of the system was determined with the sample of RP-HPLC Method for. replicates of sample solution containing 180 mg of Bempedoic acid and 10 mg Ezetimibe were injected and peak areas were measured and %RSD was calculated. is was repeated for five times :result are shown in; **(Table No : 22)& (Fig No :33,34).**

- **Application of proposed method for analysis of Repeatability:**
  Average weight of tablet sample (equivalent to 180 mg of Bempedoic acid and 10 mg Ezetimibe) were weighed and transfered to 50 ml volumetric flask & diluent was added

to make up the volume. Sonicated for 10 min with occasional swirling. The above solution was filtered through 0.45µm membrane filter 0.1 ml of this solution diluted upto 10 ml with diluent.

4.10.4 Precision:

Precision of an analytical method is the degree of agreement among Individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Also, the results obtained were subjected to one way ANOVA and within-day mean square and between-day mean square was determined and compared using F-test. **(Fig No :35)**

- **Result of Intra day and Inter day Precision studies on RP-HPLC method for Bempedoic acid and Ezetimibe**

4.10.4.1 Intra-day precision:

Sample solutions containing 180 mg of Bempedoic acid and 10 mg three different concentration(72µg/ml,108µg/ml,144µg/ml)Ezetimibe and (4µg/ml,6µg/ml,8µg/ml) Bempedoic acid and Ezetimibe were analyzed three times on the same day and %R.S.D was calculated. The Result are shown in; **(Table No.23)& (Fig No :36-41).**

4.10.4.2 Inter-day precision:

Sample solutions containing 180 mg of Bempedoic acid and 10 mg three different concentration (72µg/ml,108µg/ml,144µg/ml) Ezetimibe and (4µg/ml,6µg/ml,8µg/ml) Bempedoic acid and Ezetimibe different days and % R.S.D was calculated. It is usually expressed as standard deviation or relative standard deviation. The Result are shown in; **(Table No.23)& (Fig No :42-44).**

- **Acceptance criteria:**
  The Relative Standard Deviation should not be more than 2% for test
- **Preparation of standard stock solution:**
  180 mg of Bempedoic acid and 10 mg Ezetimibe working standards were weighed and transfered to 50 ml volumetric flask & diluent was added to make up the volume. 0.1 ml of this solution diluted upto 10 ml with diluent.

5. Robustness:

The mobile phase composition was changed in (±1 ml/ min^{-1}**)** proportion and the flow rate was **(Fig No:45,46)** of methanol: 0.05 % OPA (75:25%) in the mobile phase composition (±1ml/ min^{-1}**)** and the change in detection wavelength (±1 ml/ min^{-1}**)** and the effect of the results were examined**.(Fig No: 47,48)**and **(Fig No:49,50)** it was performed using 72 µg/ml and 4 µg/ml

solution of Bempedoic acid and Ezetimibe in triplicate. The Result are shown in; **(Table No:24 ,Table No.25).**

4.10.5 Detection Limit

Based on the S.D. of the response and the slope of calibration curve, the detection limit (DL) was calculated as,

DL = $\frac{3.3\sigma}{S}$

Where,

σ = the S.D. of the y-intercepts of regression lines.

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

The result are shown in: **(chapter:5).**

4.10.6 Quantitation Limit

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

QL = $\frac{10\sigma}{S}$

Where,

σ = the S.D. of the y-intercepts of regression lines.

S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

The result are shown in **(chapter :5).**

4.11 Analysis of marketed formulation

To determine the content of Bempedoic acid and Ezetimibe in Formulation (label claim 180 mg of Bempedoic acid and 10 mg Ezetimibe), take 236 mg of formulation dissolved in 50 ml of Methanol, To ensure complete extraction it was sonicated for 15 min. 0.4mL of supernatant was then diluted up to 10 mL with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted**. (Fig No: 51)**.

Regression equation was generated using peak areas of standard solutions. Using the regression equation and peak area of the sample the amount of Bempedoic acid and Ezetimibe in the sample was calculated. The amount of Bempedoic acid and Ezetimibe per tablet was obtained from the regression equation of the calibration curve as described in analysis of formulation are shown in **(Table No.26).**

5. RESULT AND DISCUSSION:

5.1. Preliminary studies on Bempedoic acid and Ezetimibe.

5.1.1. Melting point.

The procured reference standard of Bempedoic acid and Ezetimibe were found to melt in the range of 221^0C and 163^0C respectively.

5.1.2. Solubility

The drug was found to be,

Bempedoic acid

- Freely soluble in, methanol.
- Poorly soluble in water.

Ezetimibe:

- Practically insoluble in water,
- freely soluble in Ethanol and Methanol

5.1.3. UV Spectroscopy

UV absorption of 10 µg/mL solution of Bempedoic acid and Ezetimibe in methanol was generated and absorbance was taken in the range of 200-400 nm. λmax of Bempedoic acid and Ezetimibe in Methanol was found to be 236 nm and 227nm respectively.

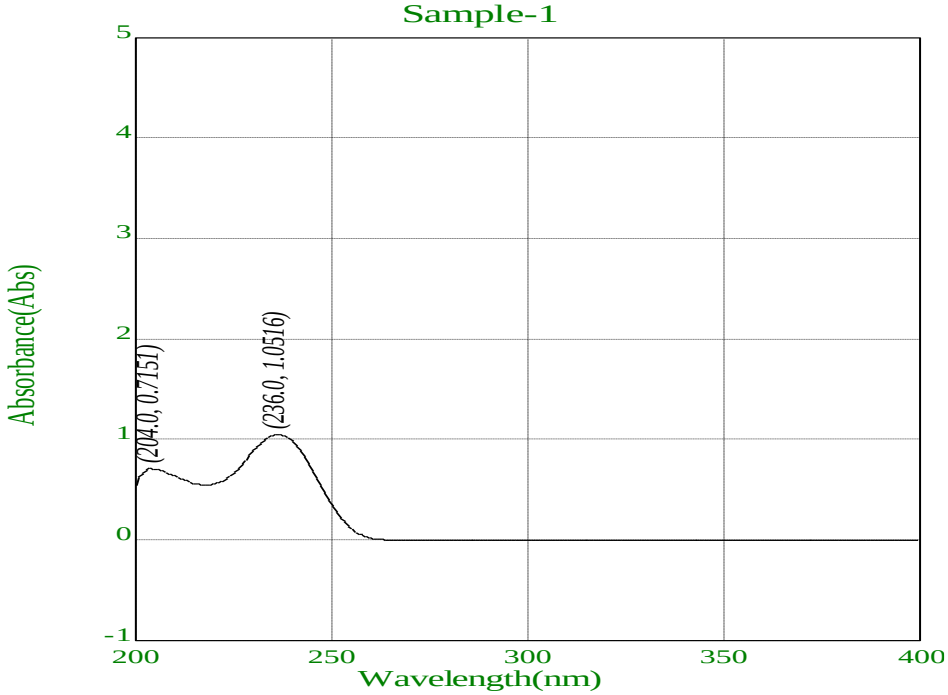


Fig No.6: UV Spectrum of Bempedoic acid

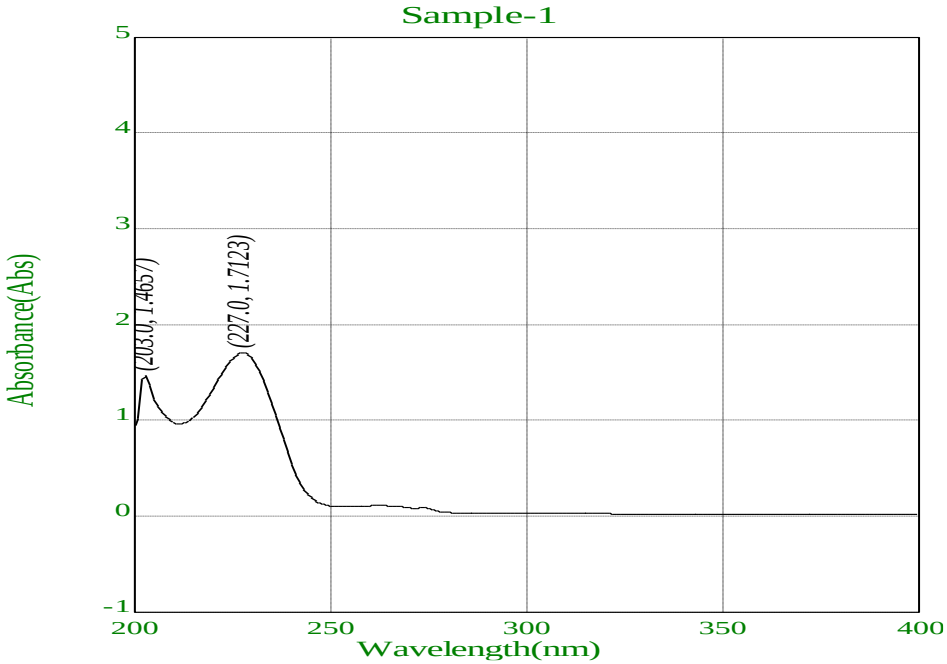


Fig No.7: UV Spectrum of Ezetimibe

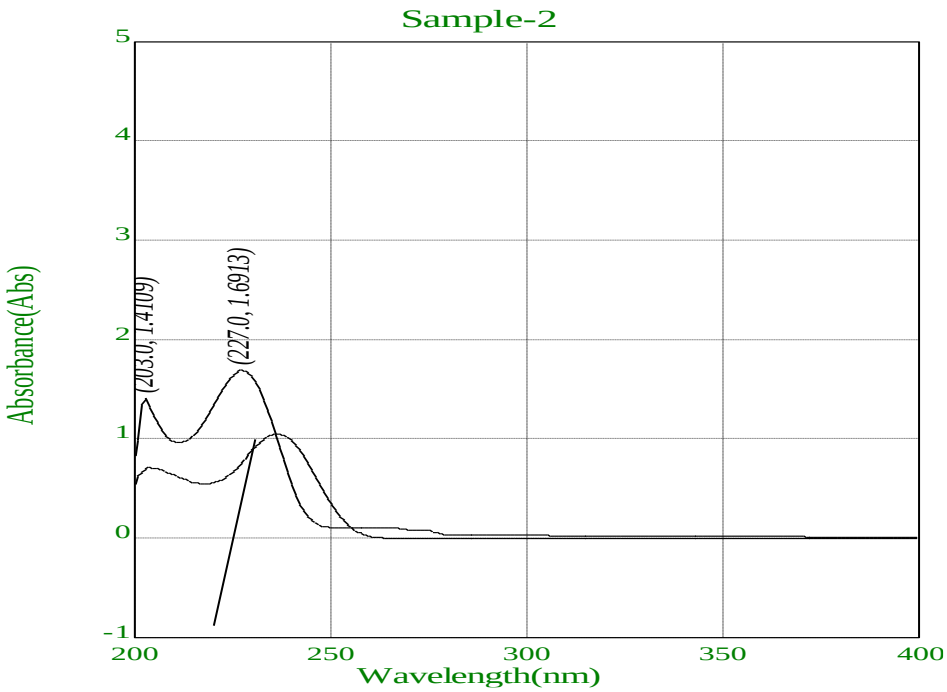


Fig.8: Iso-absorptive point of Bempedoic acid and Ezetimibe

5.1.4. Studies on the chromatographic behavior of Bempedoic acid and Ezetimibe

TABLE NO-12: Chromatographic behavior of Bempedoic acid and Ezetimibe mobile phase of various compositions.

Sr No.	Mobile Phase	Retention Time (min)		Remark
		BPD	ETM	
1.	Methanol : (0.05% OPA)(80:20 %)v/v) 233 nm	2.143	4.186	No sharp peak
2	Methanol : Water 0.05%OPA(70 : 30%v/v)PH3,233 nm flow 1 ml/min	2.199	7.005	No Sharp peak
3	Methanol : Water 0.05%OPA(50 : 50%v/v)PH3,233 nm,flow 1ml/min	2.221	2.431	No peak
4	Methanol : Water 0.05%OPA (90 : 10%v/v)PH3 233 nm,1 ml/min	2.061	3.107	No sharp peak
5	Methanol: water 0.05% OPA(75:25% v/v)PH 233 nm,1 ml/min	2.165	5.099	Sharp Peak obtain

Thus, from the above, it has been observed that, using mobile phase of Methanol + (0.05%OPA) (75+25% v/v)233 nm,1ml, pH 3 gave adequate retention time at 2.165 min and 5.099 min. with good peak shape (Theoretical plates of 5845 of Bempedoic acid & 9572 of Ezetimibe.

Chromatogram of Trial 1:

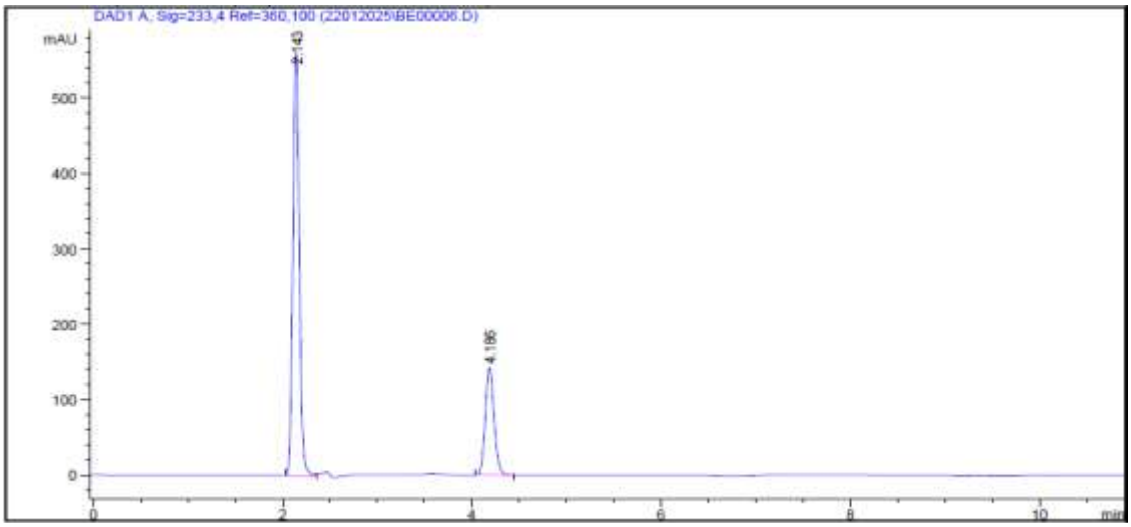


Fig.9: Representative Chromatogram of Bempedoic acid and Ezetimibe using Methanol: 0.05%OPA in Water (80:20 % v/v)) as mobile phase, showing the no sharp peak.

Chromatogram of Trial 2:

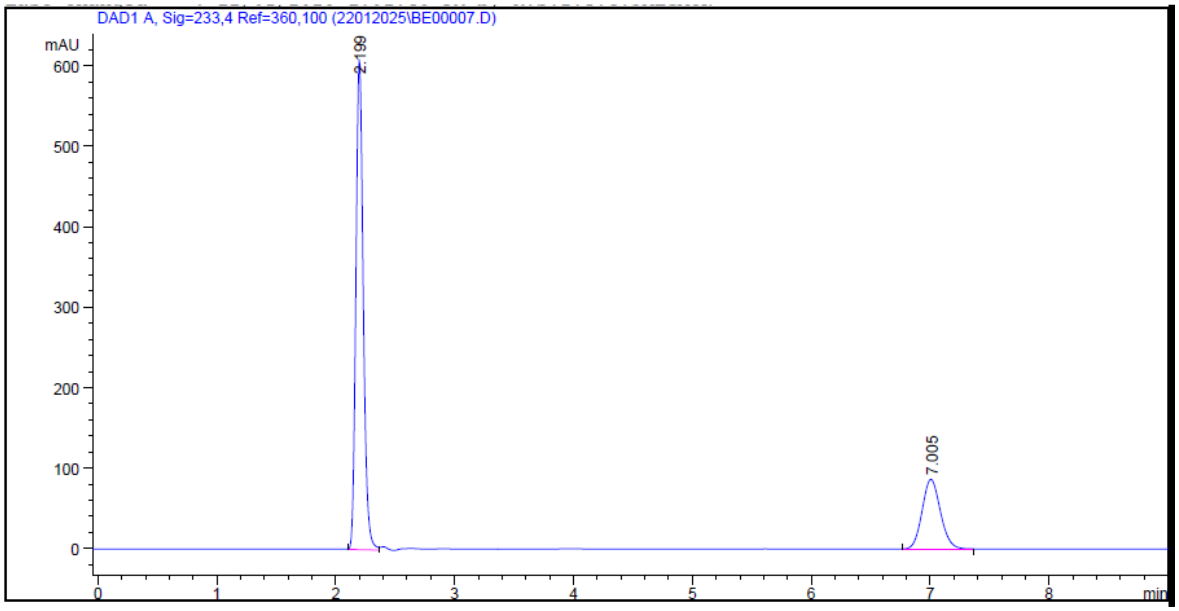


Fig.10: Representative Chromatogram of Bempedoic acid and Ezetimibe using Methanol : Water 0.05%OPA (70 : 30%v/v)PH3 1 flow rate as mobile phase, showing retention time 2.199 min & 7.005 min.

Chromatogram of Trial 3:

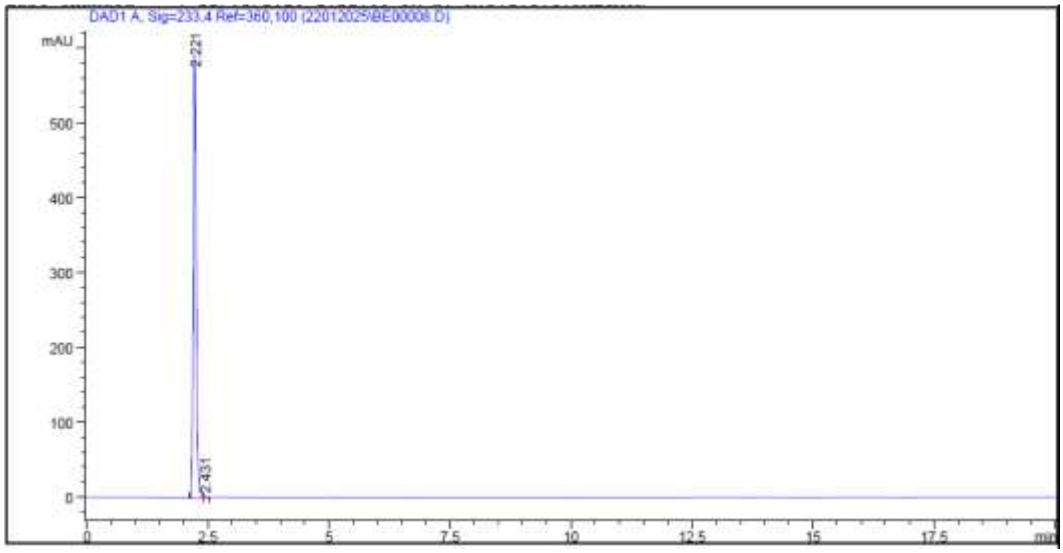


Fig.11: Representative Chromatogram of Bempedoic acid and Ezetimibe using Methanol: Water 0.05%OPA (50: 50%v/v) PH3 as mobile phase, showing no sharp peak 2.221min.

Chromatogram of Trial 4:

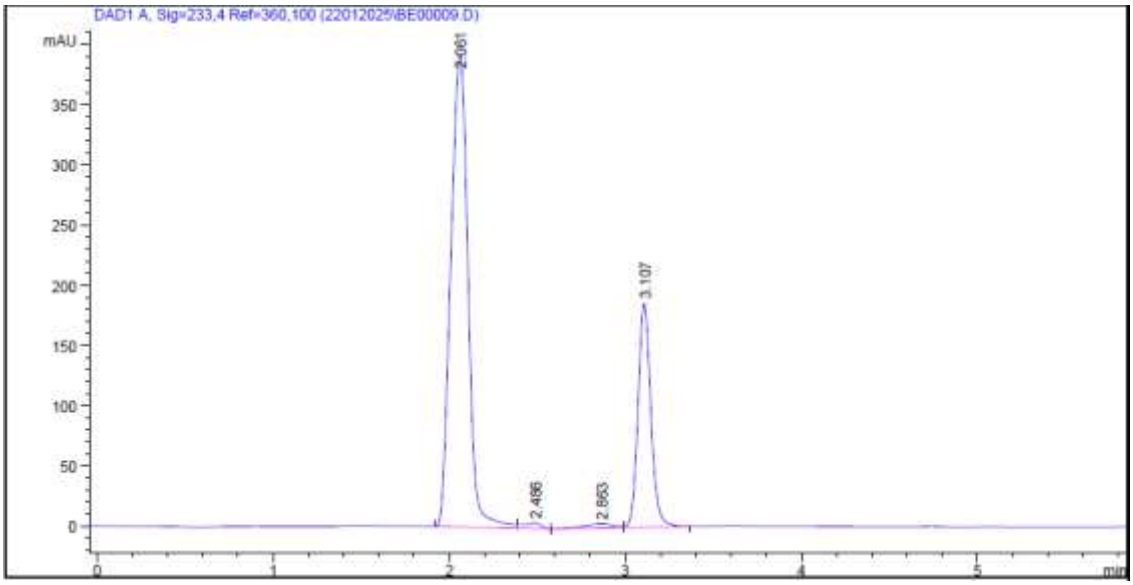


Fig.12: Representative Chromatogram of Bempedoic acid and Ezetimibe using Methanol: Water 0.05% OPA (90: 10%v/v) PH 3 as mobile phase, showing no sharp peak 2.061 min and 3.107 min.

Chromatogram of Final Graph 5:

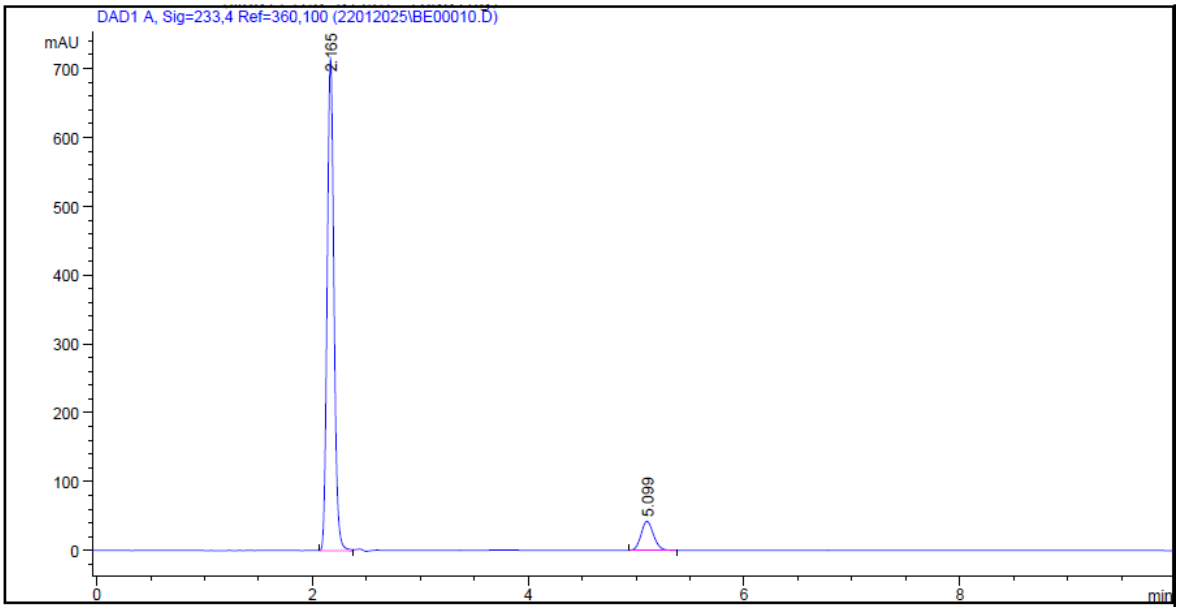
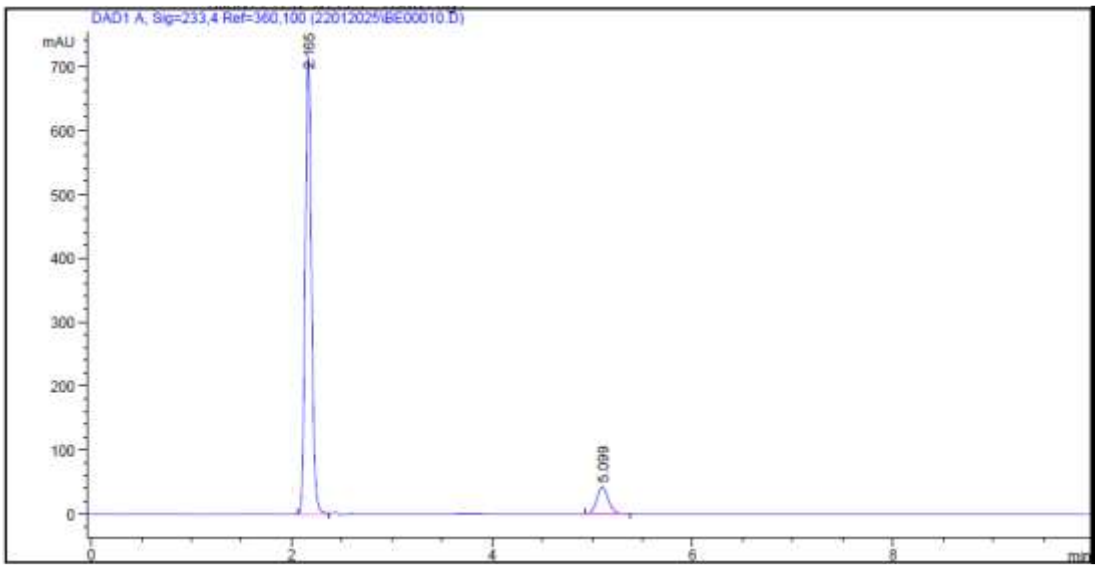


Fig.13: Representative Chromatogram of Bempedoic acid and Ezetimibe using Methanol + acidic water(0.05%OPA)(75+25% v/v)233 nm,1ml as mobile phase, showing retention time 2.165 min and 5.099 min.sharp peak were obtain, so this method are selected.

- **The final chromatographic conditions selected were as follow:**

- Analytical column : Agilent C18 Column (250mm x 4.6mm, 5µm partical size).
- Injection volume : 20µl
- Flow rate : 1 ml/min
- Mobile phase : Methanol: (0.05% OPA),75:25 %
- Detection : 233 nm
- Run Time : 10 min



FigNo.14: Chromatogram of standard Combination of Bempedoic acid and Ezetimibe.

Table.No.13. Details of chromatogram of standard Combination containing Bempedoic acid and Ezetimibe

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	2.165	3126.3349	5845	0.88	0.0000
2	5.099	338.5495	9572	0.85	18.21

In the standard mixture of Bempedoic acid and Ezetimibe theoretical plates were found above 2000 i.e. for Bempedoic acid and Ezetimibe 5845 and 9572 at minimum RT 2.165 and 5.099 respectively**.**

5.1.5 Calibration experiment

➢ **RP-HPLC Method :**

The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 36-180µg/mL for Bempedoic acid and 2-10 µg/mL for Ezetimibe (**Table No:14,and Table No:15)**depict the calibration data of Bempedoic acid and Ezetimibe The respective linear equation for

Bempedoic acid was y = 70.18X+1178 and Ezetimibe equation y = 101.6X+9.032 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Bempedoic acid and Ezetimibe is depicted in(**FigNo.15 and Fig No.16).**

Table No 14: Linearity data for Bempedoic acid

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
RP-HPLC Method	36	3707.69	3708.59	3708.14	0.64	0.02
	72	6141.3222	6248.57	6194.95	75.84	1.22
	108	8800.4755	8895.81	8848.15	67.42	0.76
	144	11184.7	11228.8	11206.75	31.18	0.28
	180	13860.5	13809.2	13834.85	36.27	0.26
	Equation		y = 70.18x+1178			
	R^2		0.999			

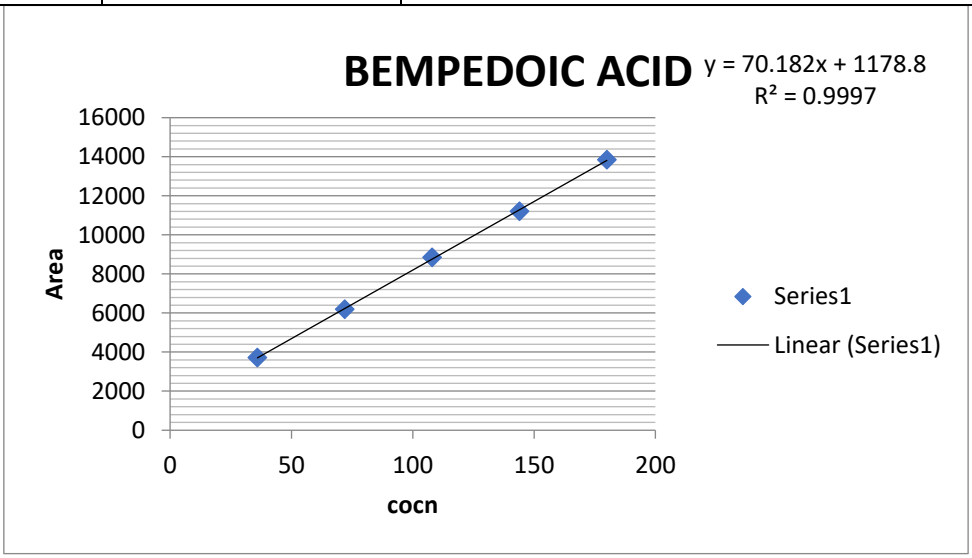


Fig.No.15: Calibration curve of Bempedoic acid

The RP-HPLC Method for respective linear equation for Bempedoic acid was y = 70.18x +1178 where x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Bempedoic acid is depicted in **Fig 14.**

Table No 15: Linearity data for Ezetimibe

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
RP-HPLC Method	2	208.2513	210.727	209.49	1.75	0.84
	4	420.0191	420.3393	420.18	0.23	0.05
	6	620.2708	623.0107	621.64	1.94	0.31
	8	814.905	814.8101	814.86	0.07	0.01
	10	1024.976	1032.475	1028.73	5.30	0.52
	Equation		y = 101.6x+9.032			
	R^2		0.999			

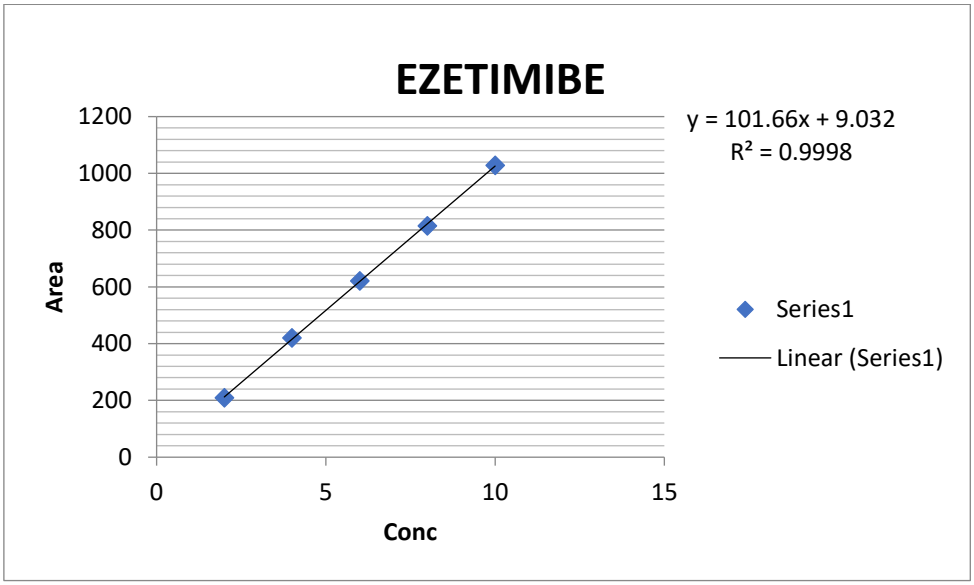


Fig.No.16: Calibration curve o Ezetimibe

The RP-HPLC method for respective linear equation for Ezetimibe was y = 101.6X+9.032 sswhere x is the concentration and y is area of peak. The correlation coefficient was 0.999. The calibration curve of Ezetimibe is depicted in **Fig15.**

5.2. Analytical of Method Validation:

1. Linearity:

From Bempedoic acid standard stock solution, different working standard solution (36-180 µg/ml) were prepared in mobile phase Likewise from Ezetimibe standard stock solution different working standard solution (2-10µg/ml) were prepared in mobile phase 20 µl of sample solution was injected into the chromatographic system using fixed volume loop injector. Chromatograms were recorded. The area for each conc entration were recorded **(Table No. 16,18).** The Calibration curves are shown in **Fig. No.28,29).**

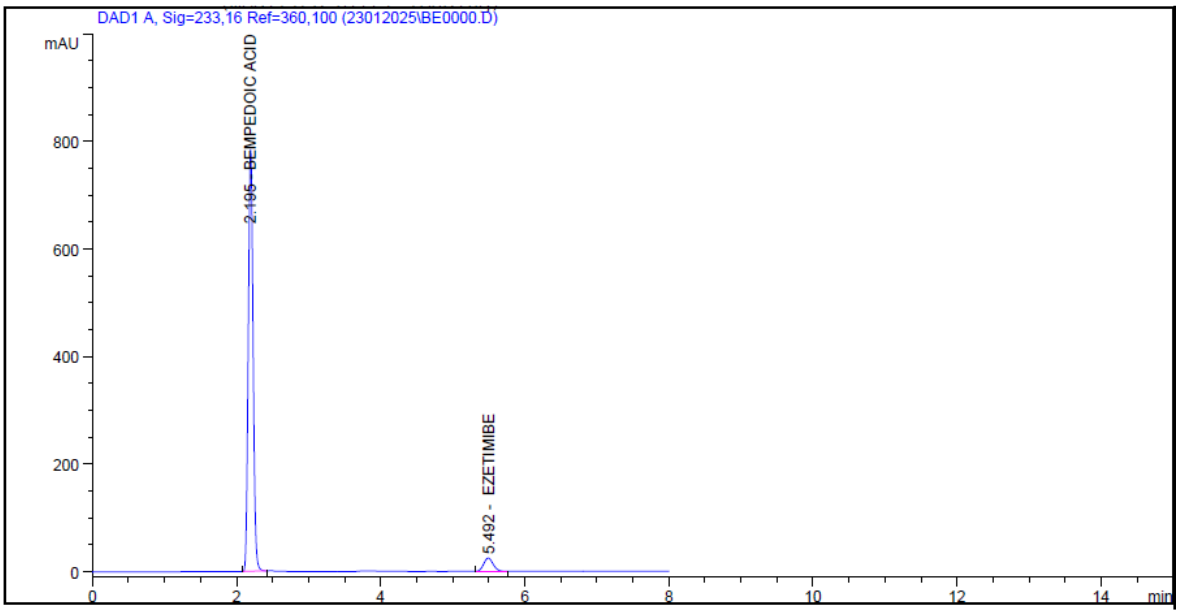


Fig.No.17. Chromatogram of linearity

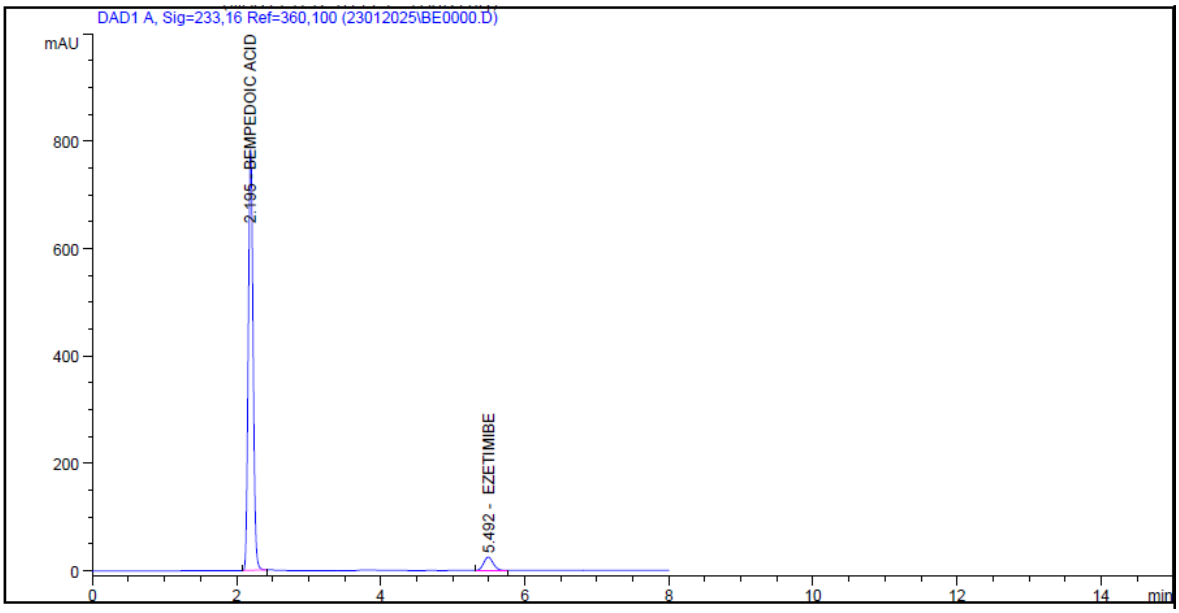


Fig.No.18. Chromatogram of linearity 2+36 mcg-01

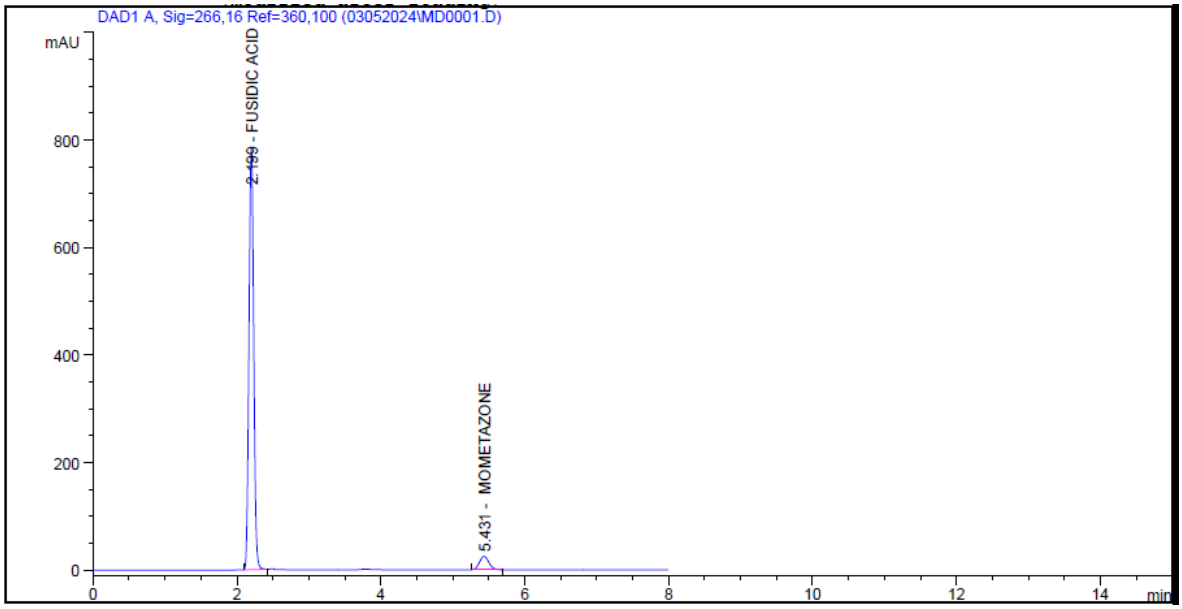


Fig.No.19. Chromatogram of linearity 2+36 mcg-02

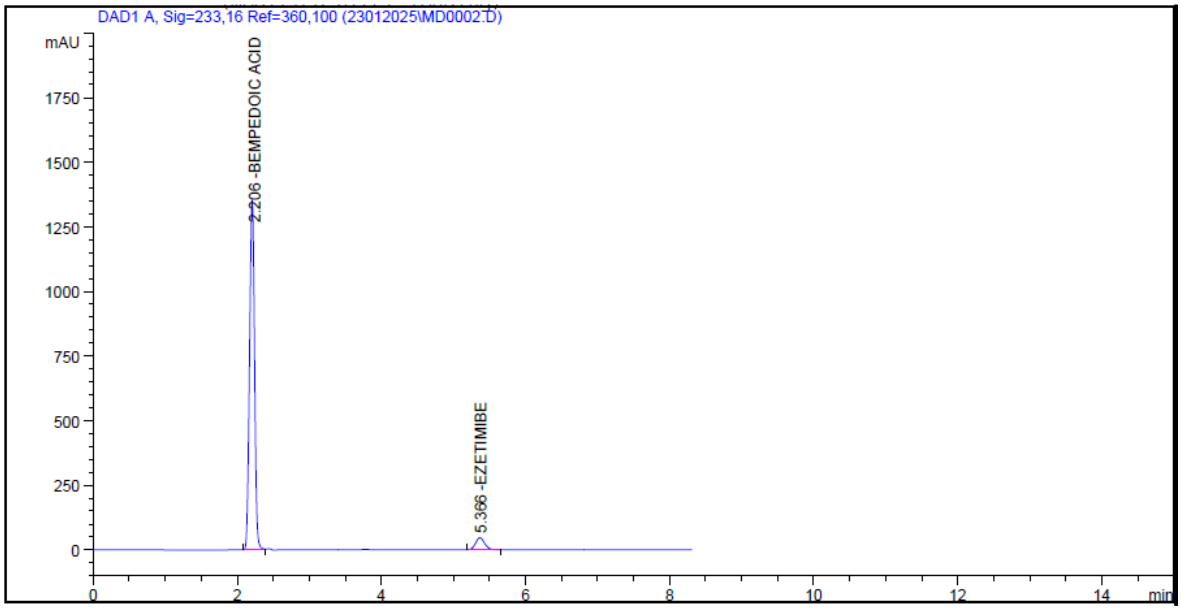


Fig.No.20. Chromatogram of linearity 4+72 mcg-01

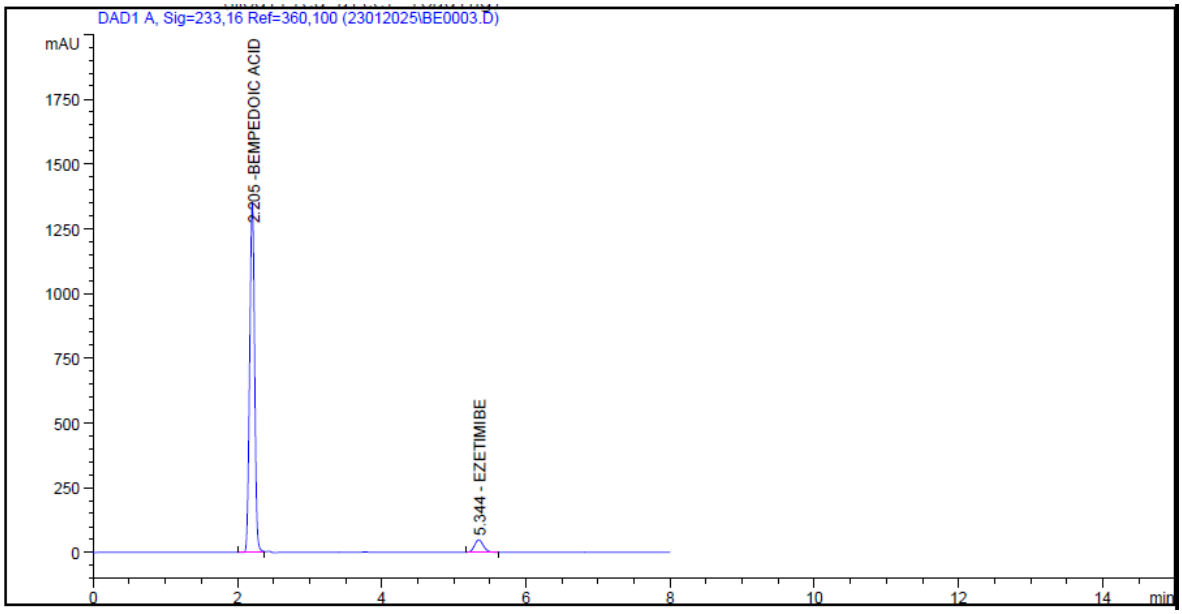


Fig.No.21. Chromatogram of linearity 4+72 mcg-02

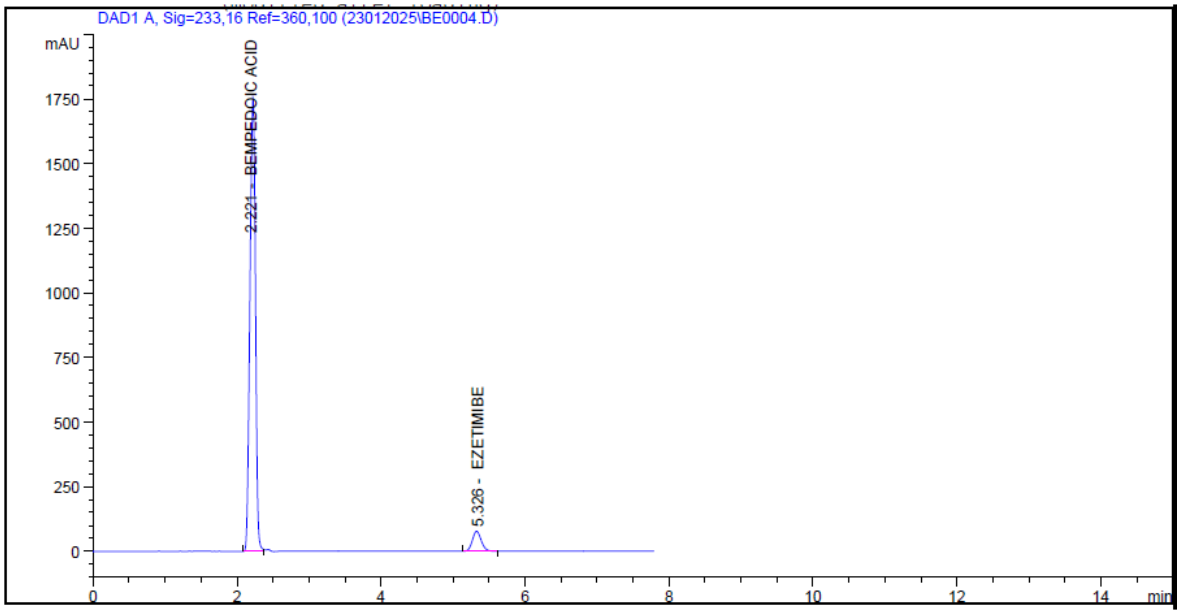


Fig.No.22. Chromatogram of linearity 6+108 mcg-01

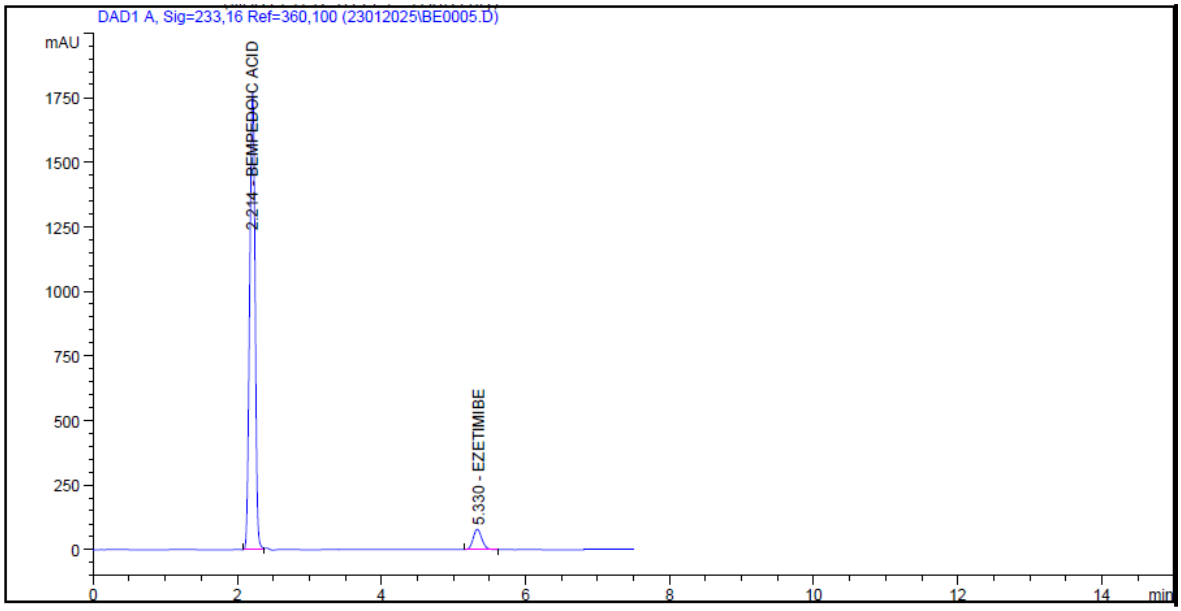


Fig.No.23. Chromatogram of linearity 6+108 mcg-02

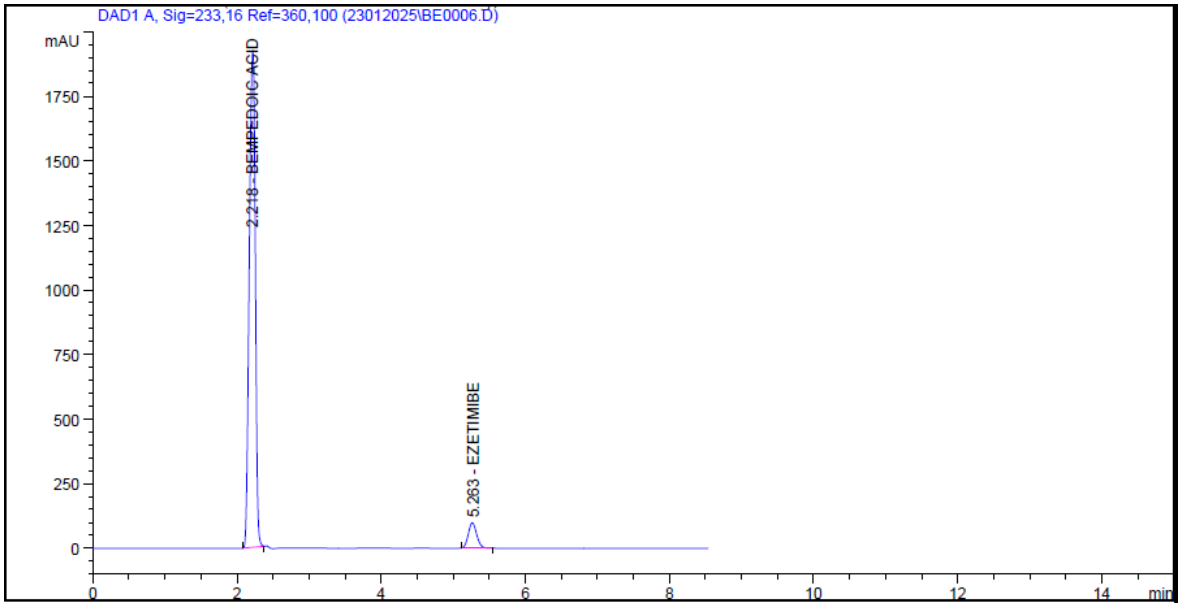


Fig.No.24. Chromatogram of linearity 8+144 mcg-01

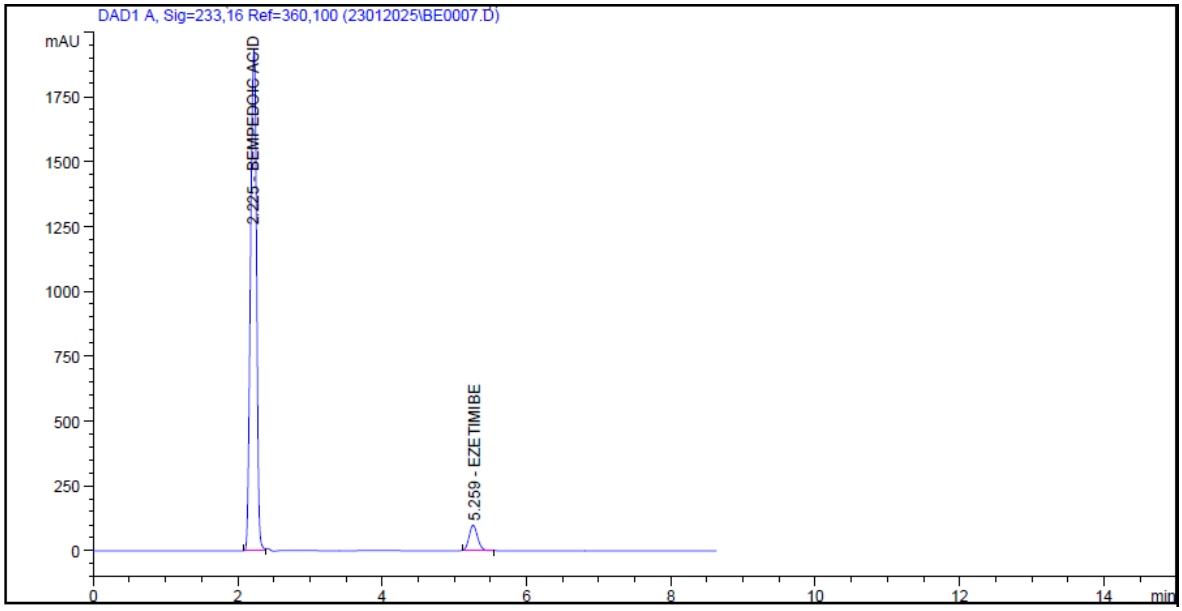


Fig.No.25. Chromatogram of linearity 8+144 mcg-02

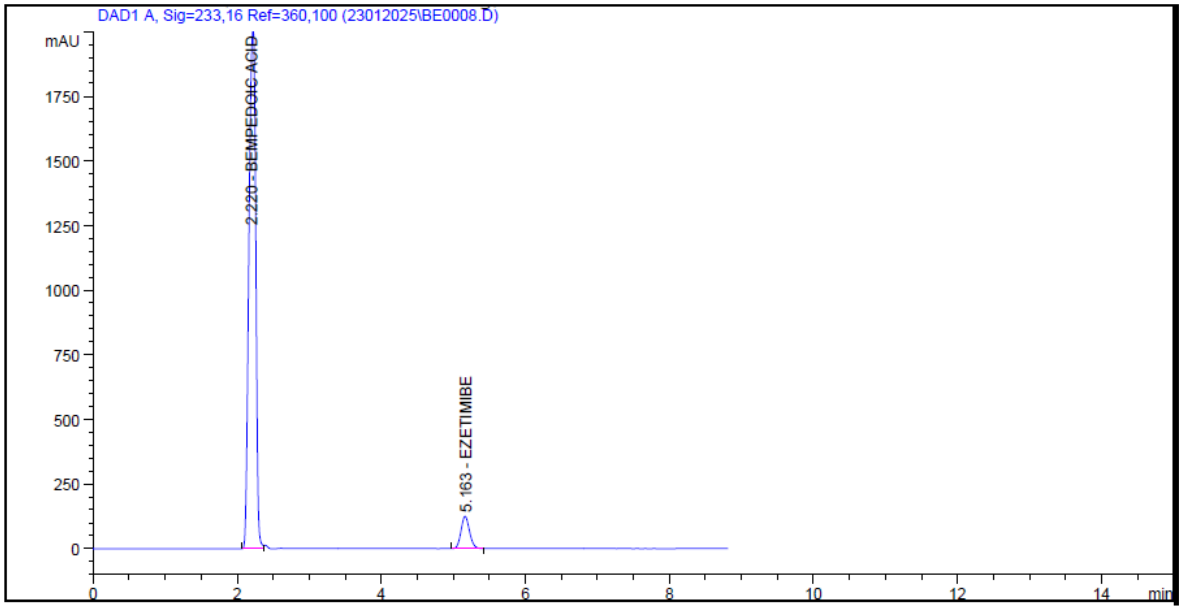


Fig.No.26. Chromatogram of Linearity 10+180 mcg-01

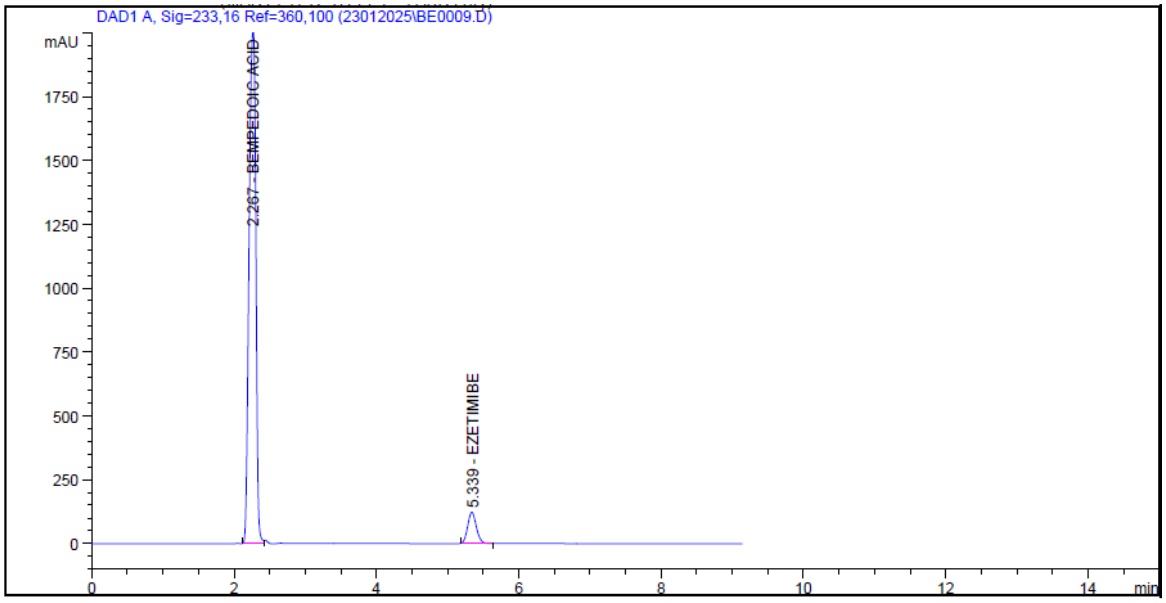


Fig.No.27. Chromatogram of Linearity 10+180 mcg-02

Table No 16. Linearity of Bempedoic acid

Concentration µg/ml	Area Bempedoic acid
36	3708.14
72	6194.55
108	8848.15
144	11206.75
180	13834.85

Fig.No.28. Calibration curve of Bempedoic acid for HPLC method

Table No 17. Regression equation data for Bempedoic acid

Regression Equation Data Y=mx+c	
Slope(m)	70.18
Intercept(c)	1178
Correlation Coefficient	0.998

Table No 18. Linearity of Ezetimibe

Concentration µg/ml	Area Ezetimibe
2	209.49
4	420.18
6	621.64
8	814.86
10	1028.73

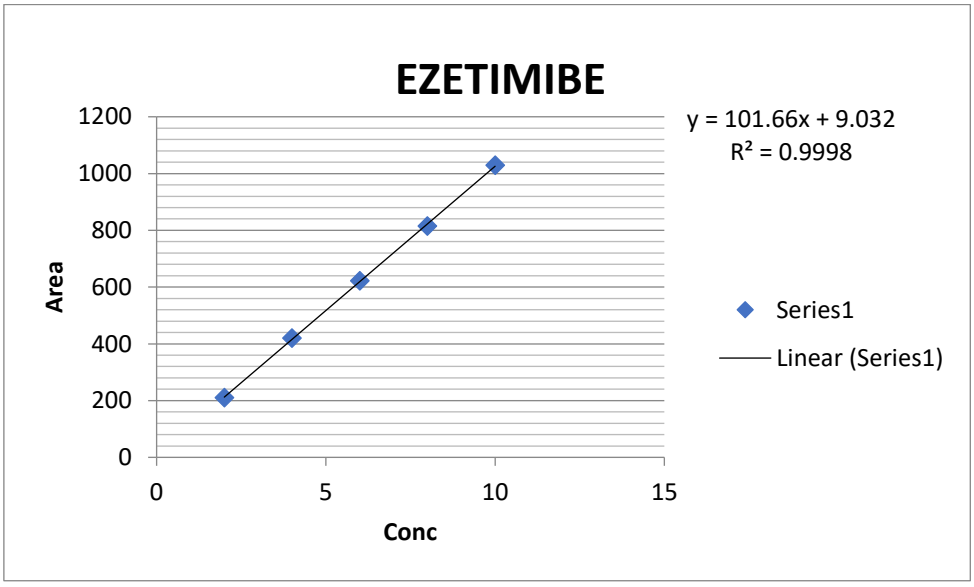


Fig No.29. Calibration graph of Ezetimibe for HPLC method

Table.19. Regression equation data for Ezetimibe

Regression Equation Data Y=mx+c	
Slope(m)	101.6
Intercept(c)	9.032
Correlation Coefficient	0.999

Linearity of of Bempedoic acid and Ezetimibe was observed in the range of 36-180 µg/ml and 2-10µg/ml. Detection wavelength used was 233 nm**. (Table No. 17,19)**

The plot should be linear passing through the origin; Correlation Coefficient should not be less than 0.999.that concluded. **. (Table. No. 16, 18)**

2. Accuracy: -

Recovery studies were performed to validate the accuracy of developed method. To pre analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed **(Table No.20).** Statistical validation of recovery studies shown in **(Table No. 21).**

Accuracy 80%

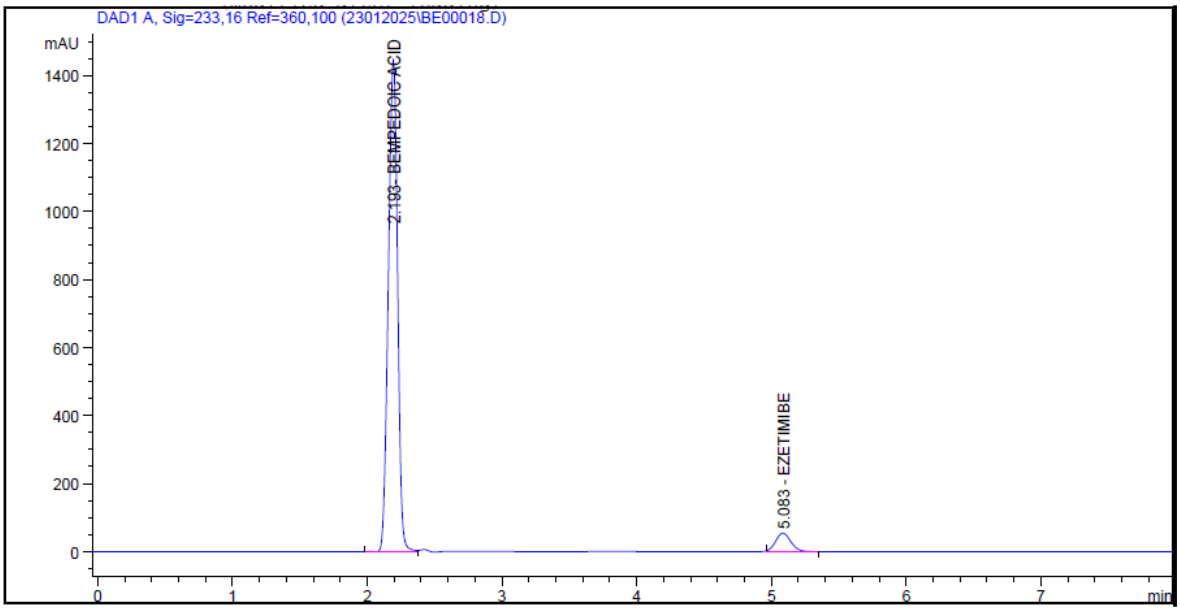


Fig.30. Chromatogram of Accuracy 80%

Accuracy 100%

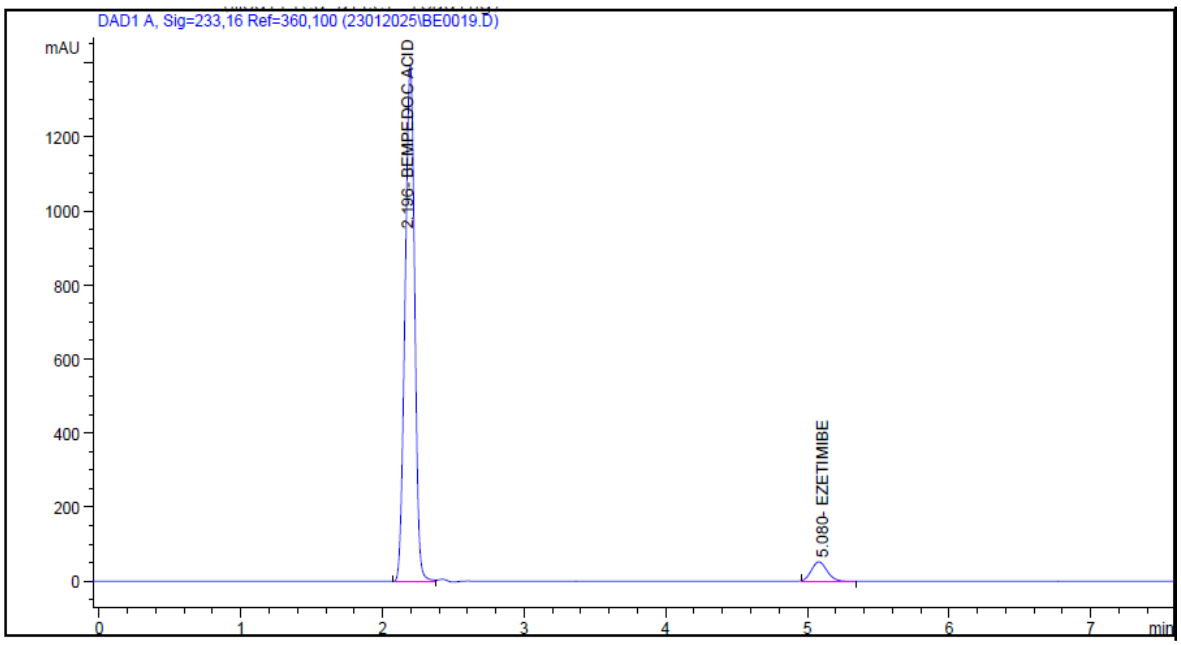


Fig.31. Chromatogram of Accuracy 100%

Accuracy 120%

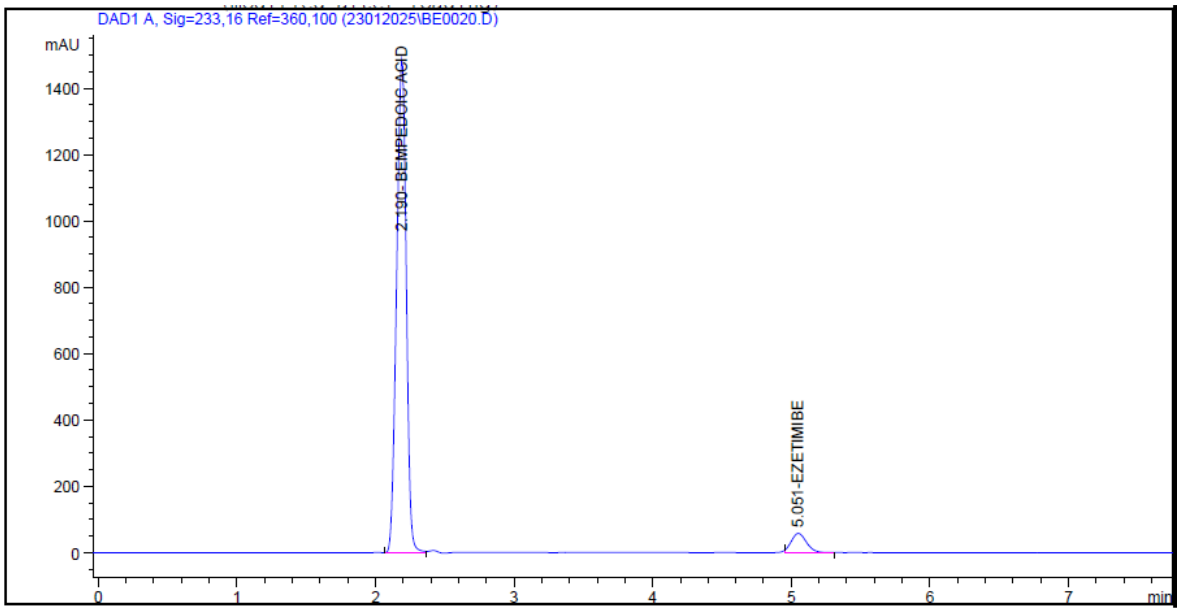


Fig.32. Chromatogram of Accuracy 120%

Table 20. Result of Recovery data for Bempedoic acid and Ezetimibe

METHOD	Drug	Level (%)	Amt. taken (µg/ml	Amt. Added (µg/ml	Area Mean* ± S.D.	Amt. recovered Mean *±S.D.	%Recovery Mean *± S.D.
RP-HPLC Method	BPD	80%	36	28.8	64.88±0.021	28.88±0.021	100.28±0.07
		100%	36	36	72.32±0.048	36.32±0.048	100.89±0.13
		120%	36	43.2	79.89±0.052	43.89±0.052	101.60±0.12
	ETM	80%	2	1.6	3.60±0.001	1.60±0.001	100.28±0.05
		100%	2	2	4.00±0.000	2.00±0.000	100.05±0.01
		120%	2	2.4	4.44±0.002	2.44±0.002	101.62±0.07

***mean of each 3 reading for RP-HPLC method**

Table.21. Statistical Validation of Recovery Studies Bempedoic acid and Ezetimibe

METHOD	Level of Recovery (%)	Drug	Mean % Recovery	Standard Deviation*	% RSD
Rp-HPLC Method	80%	BPD	100.28	0.07	0.07
		ETM	100.28	0.05	0.05
	100%	BPD	100.89	0.13	0.13
		ETM	100.05	0.00	0.00
	120%	BPD	101.60	0.12	0.12
		ETM	101.62	0.07	0.06

***Denotes average of three determinations for RP-HPLC**

Accuracy of RP-HPLC method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 98-102% **(Table No. 20,21)**.

3. System suitability parameters :(Repeatability)

To ascertain the resolution and reproducibility of the proposed chromatographic system for estimation of Bempedoic acid and Ezetimibe system suitability parameters were studied. The result shown in below **(Table No.22)**

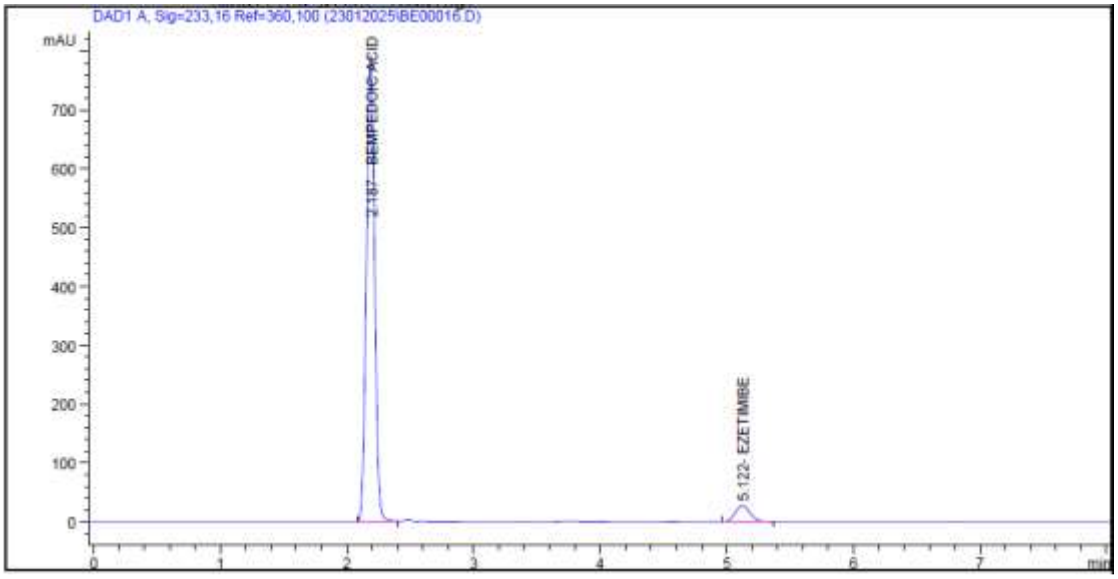


Fig No.33: Chromatogram of System suitability No (2+36 mcg) - 1

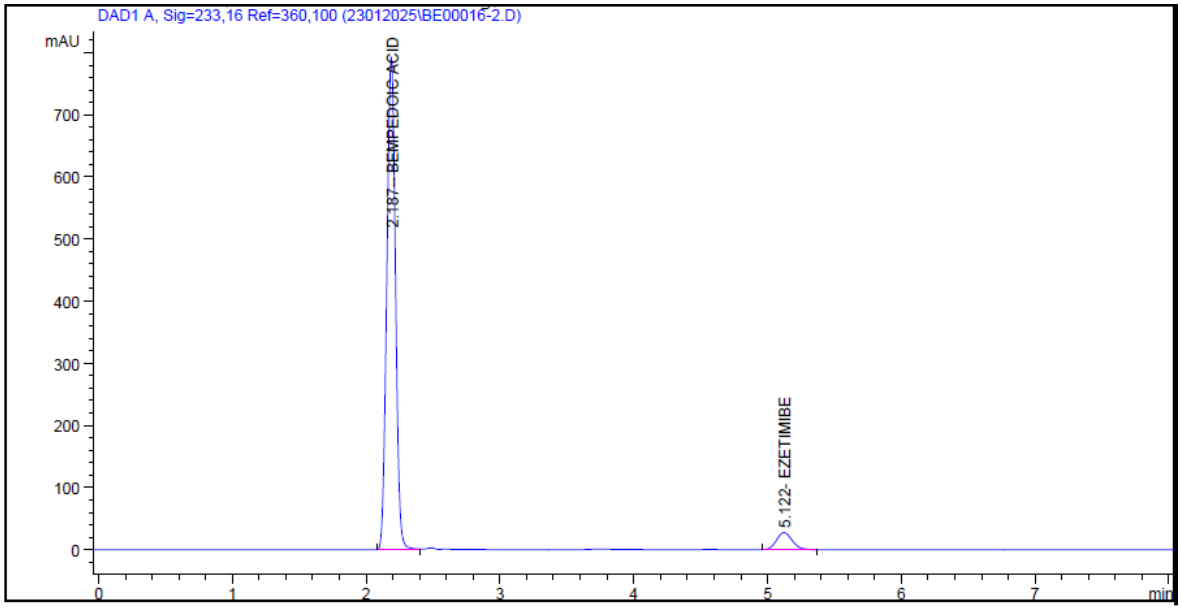


Fig No.34: Chromatogram of System suitability No (2+36 mcg)- 2

Table No.22: Repeatability studies on RP-HPLC for Bempedoic acid and Ezetimibe

Method	Concentration of Bempedoic acid and Ezetimibe (mg/ml)	Peak area	Amount found (mg)	% Amount found
RP-HPLC Method for BPD	36	3768.786	35.52	100.45
	36	3713.517	36.23	101.45
		Mean	**3441.15**	**101.45**
		SD	**39.08**	**39.08**
		%RSD	**1.04**	**1.04**
RP-HPLC Method for ETM	2	210.244	1.92	99.50
	2	212.548	1.99	99.60
		Mean	**211.40**	**99.57**
		SD	**1.63**	**3.04**
		%RSD	**0.77**	**0.77**

Repeatability studies on RP-HPLC for Bempedoic acid and Ezetimibe was found to be ,The %RSD was less than 2%, which shows high percentage amount found in between 98% to 102% indicates the analytical method that concluded .**(Table No.22)**.

4. Precision: -

The method was established by analyzing various replicates standards of Bempedoic acid and Ezetimibe. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result that concluded. The result obtained for intraday is shown in **(Table No. 23)** respectively.

Chromatogram of Precision:

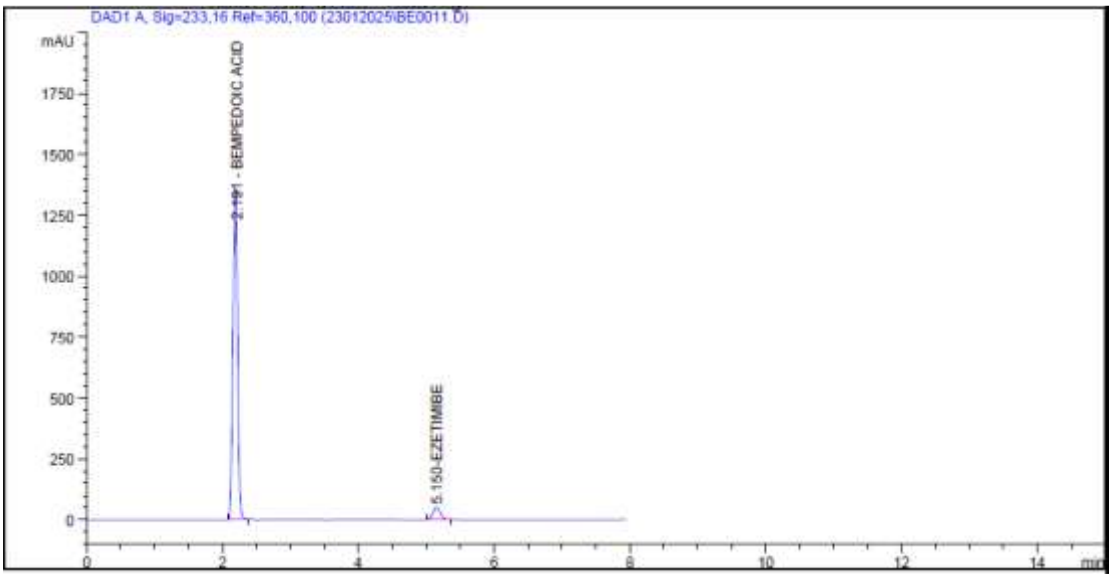


Fig No .35: Chromatogram of Precision

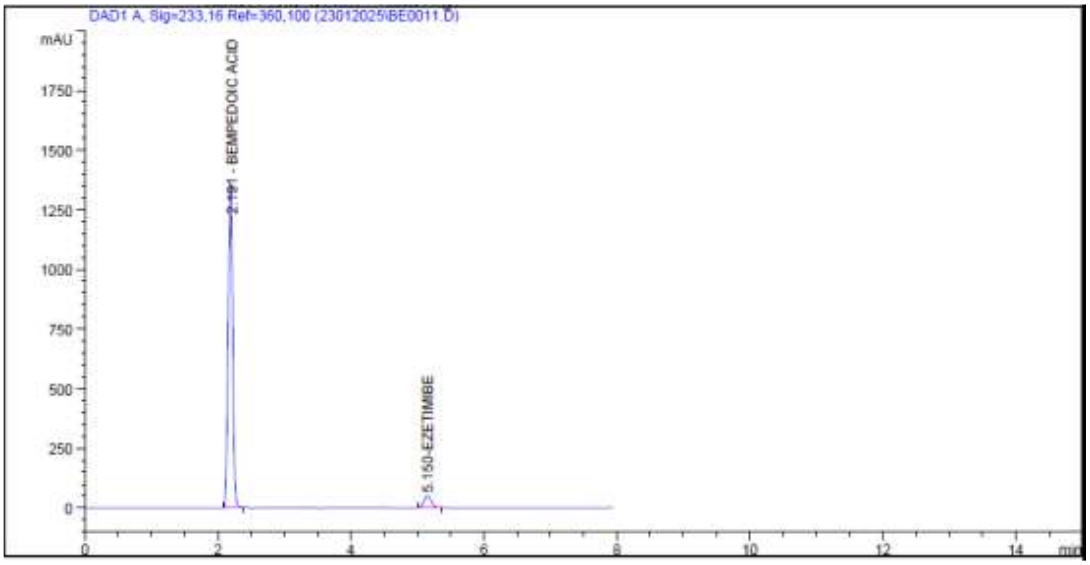


Fig No.36: Chromatogram Intra-day precision4+72 mcg-01

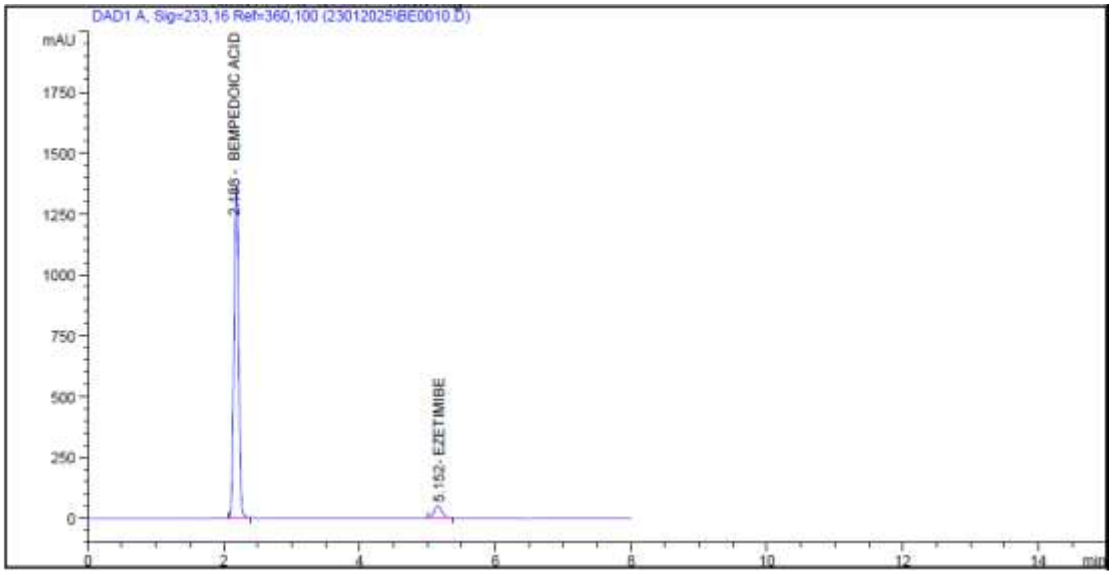


Fig No.37: Chromatogram Intra-day precision 4+72 mcg-02

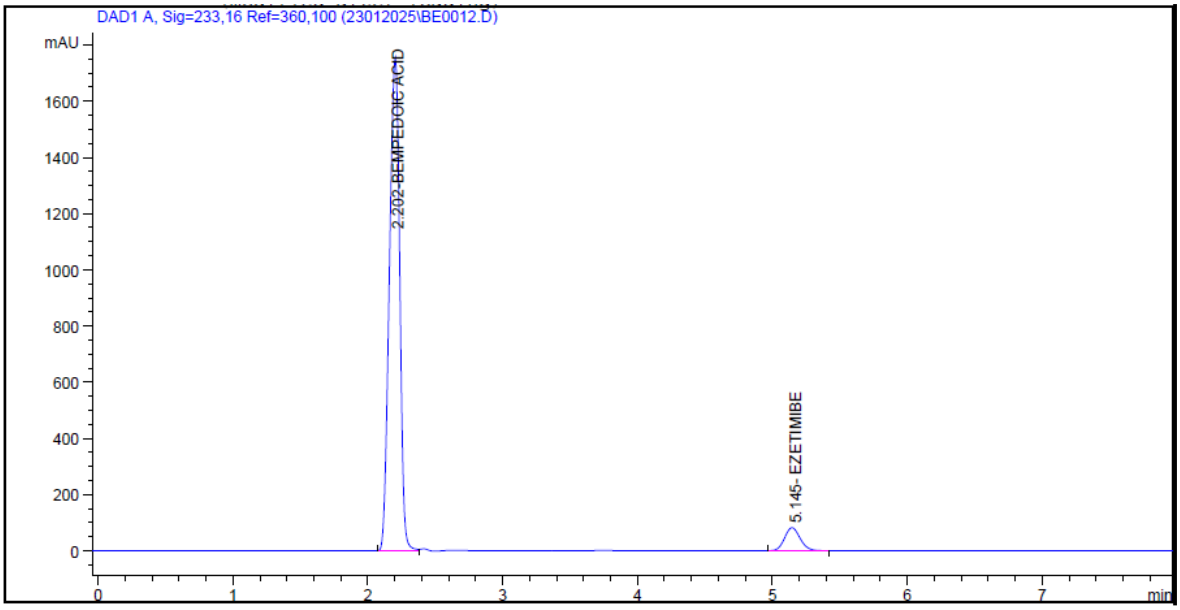


Fig No.38: Chromatogram Intra-day precision 6+108 mcg-01

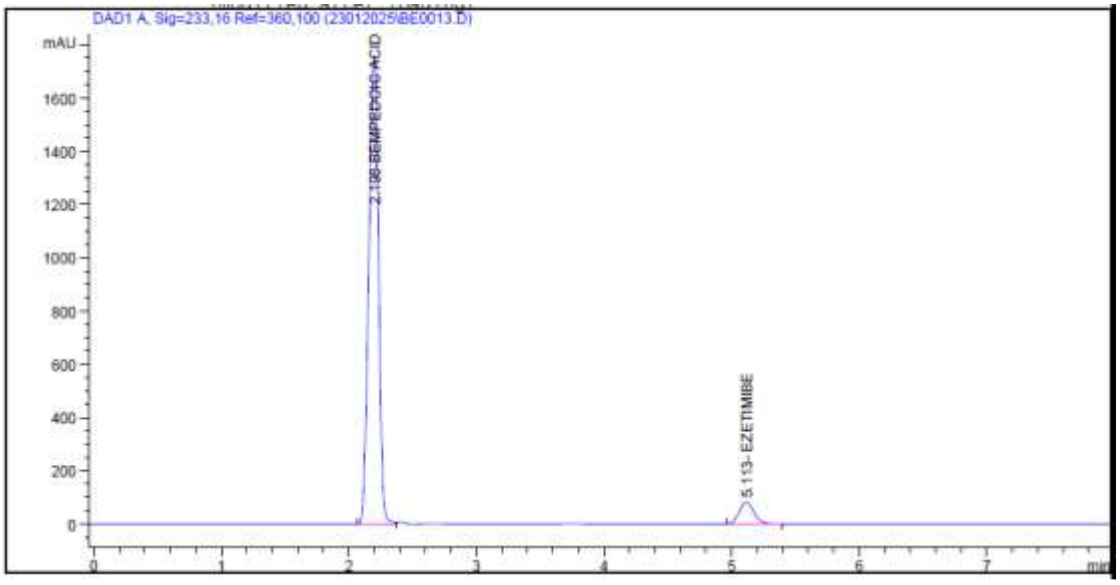


Fig No.39: Chromatogram Intra-day precision 6+108 mcg-02

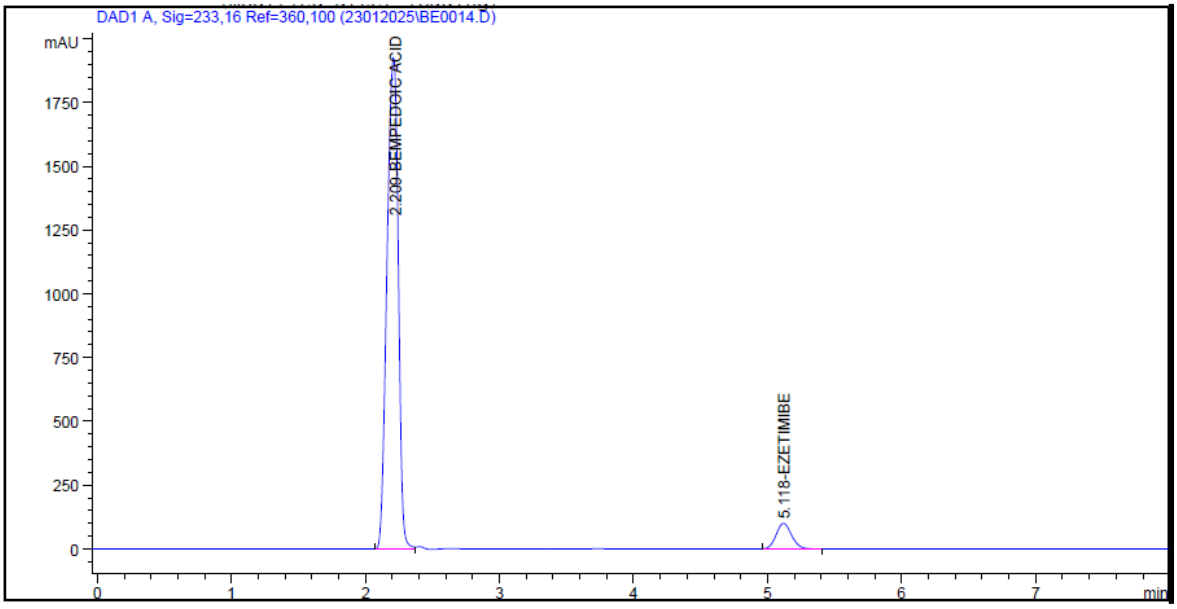


Fig No.40: Chromatogram Intra-day precision 8+144 mcg-01

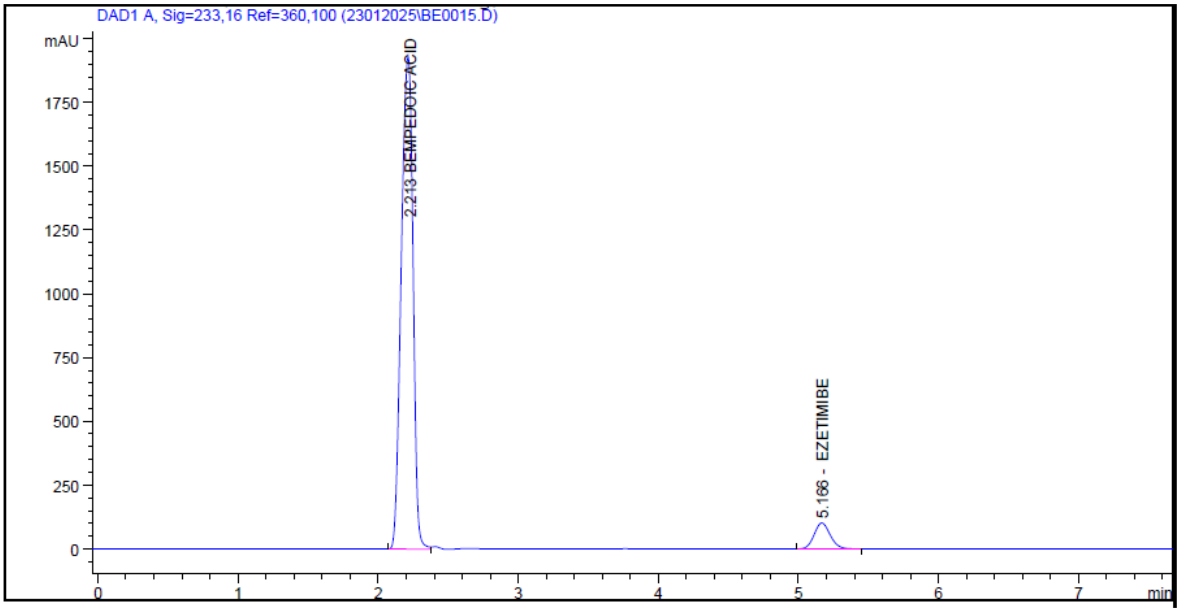


Fig No.41: Chromatogram Intra-day precision 8+144 mcg-02

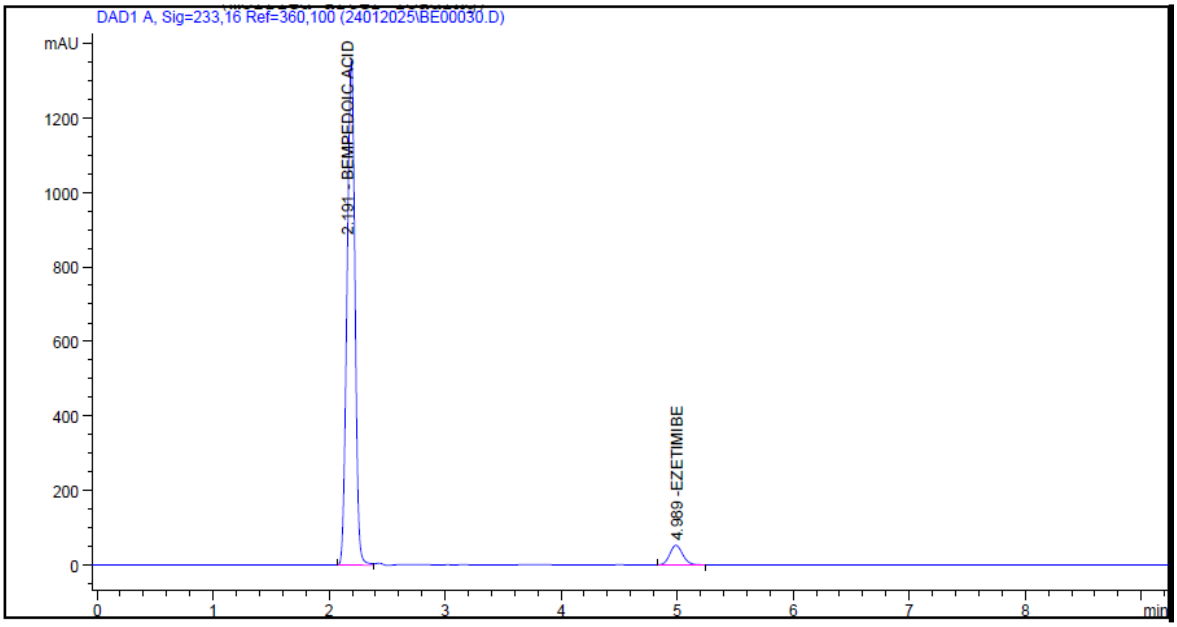


Fig No.42: Chromatogram Inter-day precision 4+72 mcg-01

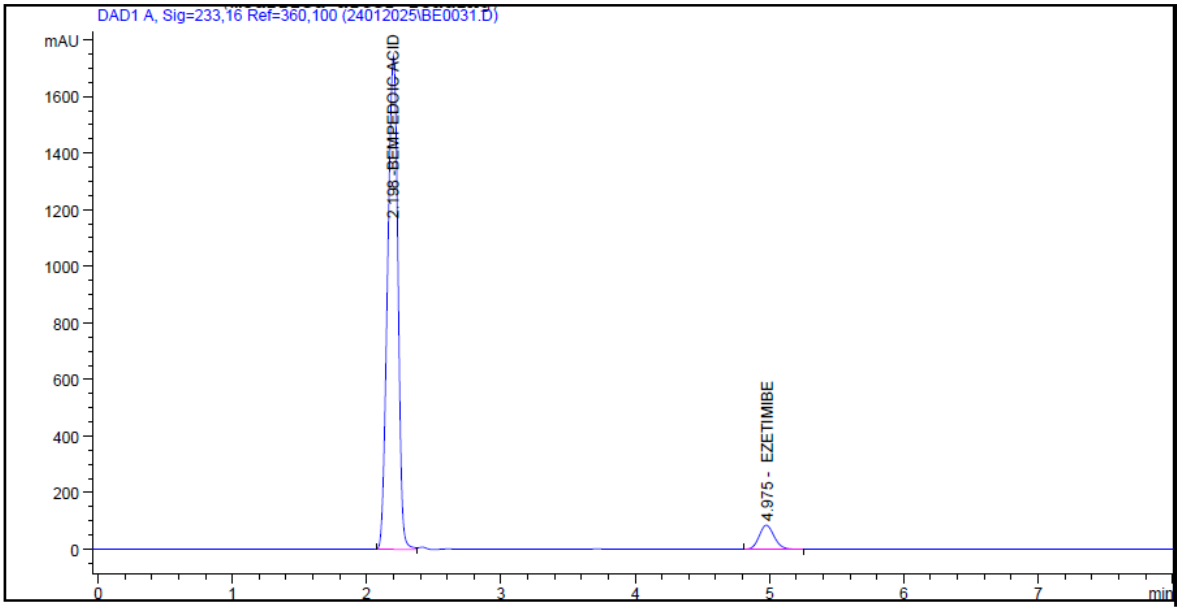


Fig No.43: Chromatogram Inter-day precision 6+108 mcg

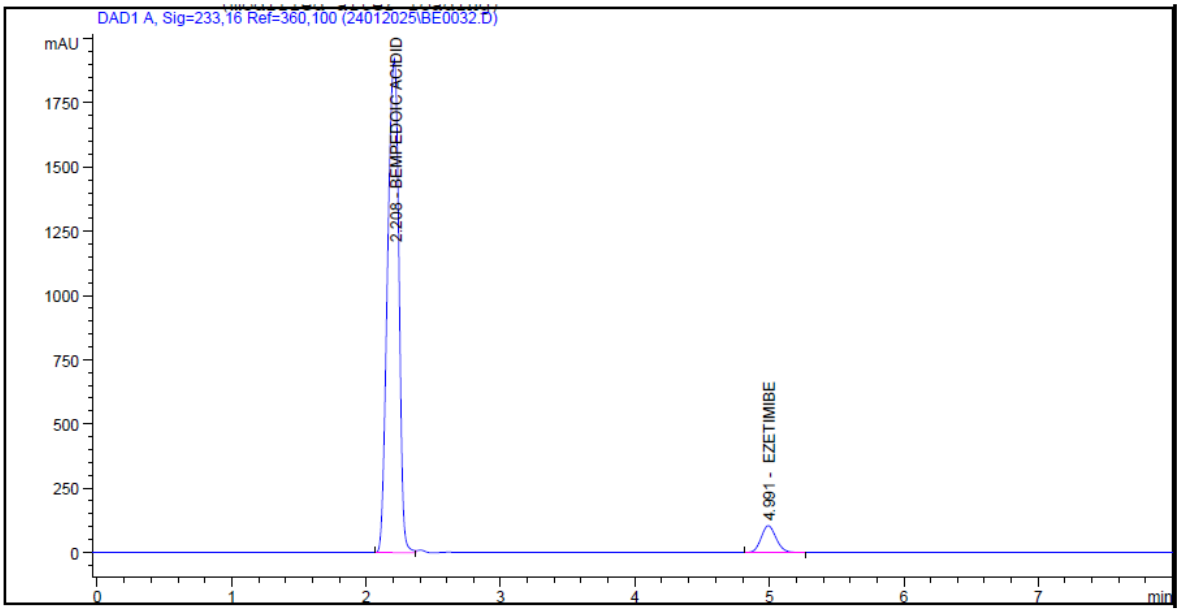


Fig No.44: Chromatogram Inter-day precision 8+144 mcg

Table No .23: Result of Intra day and Inter day Precision studies on RP-HPLC for Bempedoic acid and Ezetimibe

METHOD	Drug	Conc^{n} (µg/ml)	Intraday Precision		Interday Precision	
			Mean± SD	%Amt Found	Mean± SD	%Amt Found

Rp-HPLC METHOD	**BPD**	72	6187.07±14.30	99.13	6169.99±0.38	98.79
		108	8817.21±5.69	100.79	8819.72±1.16	100.82
		144	11243.2±11.88	99.60	11244.75±1.62	99.61
	ETM	4	421.33±0.82	101.43	418.54±2.39	100.75
		6	625.33±0.05	101.08	625.20±0.31	101.06
		8	815.96±3.03	99.26	815.79±0.48	99.24

***Mean of each 3 reading for RP-HPLC**

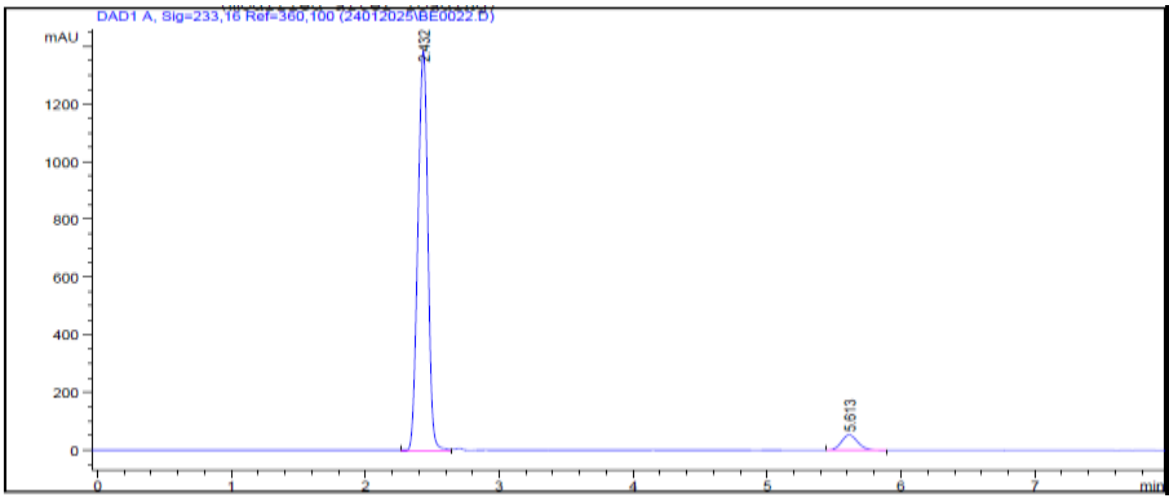
Intraday and Inter day Precision studies on RP-HPLC for Bempedoic acid and Ezetimibe which shows the high precision %amount in between 98% to 102% indicates to analytical method that concluded.

5. Robustness:

The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate, wavelength on retention time and tailing factor of drug peak was studied.

The mobile phase composition was changed in (±1 ml/min^{-1}**)** proportion and the flow rate was varied by (±1ml/min^{-1}**)**, and wavelength change (±1 ml/min^{-1}**)** of optimized chromatographic condition. The results of robustness studies are shown in **(Table No.24, 25**).Robustness parameters were also found satisfactory; hence the analytical method would be concluded.

1) Flow Rate Change 0.9 ml



FigNo.45. Chromatogram of Flow rate change 0.9ml (4+72mcg)

2) Flow Rate Change 1.1 ml

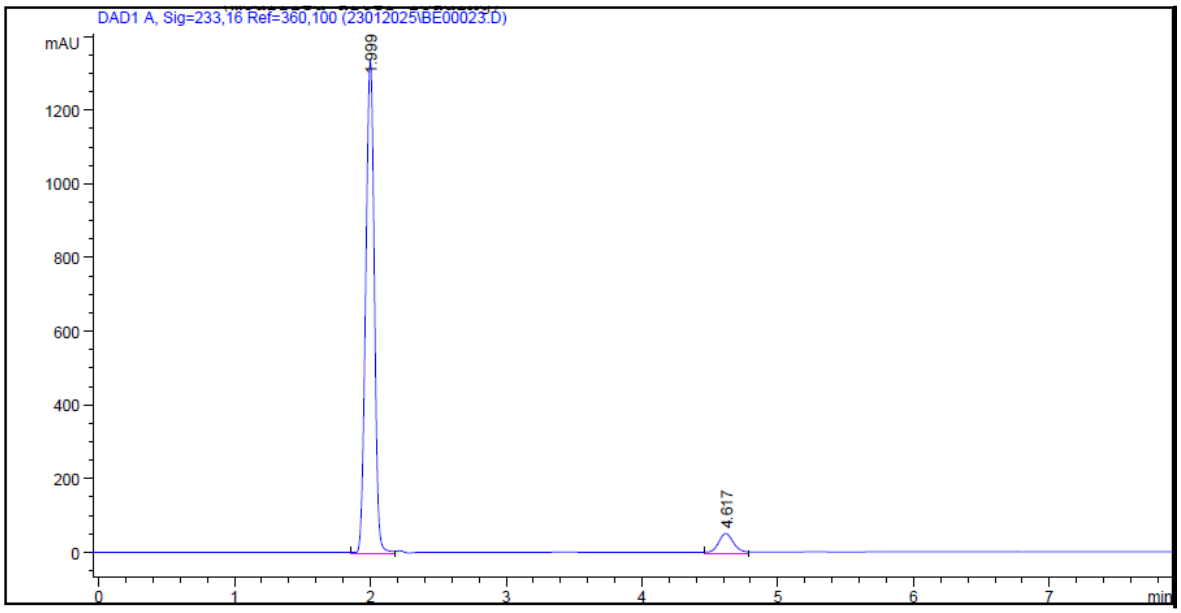


Fig No 46. Chromatogram of Flow rate change 1.1 ml (4+72 mcg)

3) Mobile phase composition Change : 74ml Methanol + 26 ml0.05%OPA

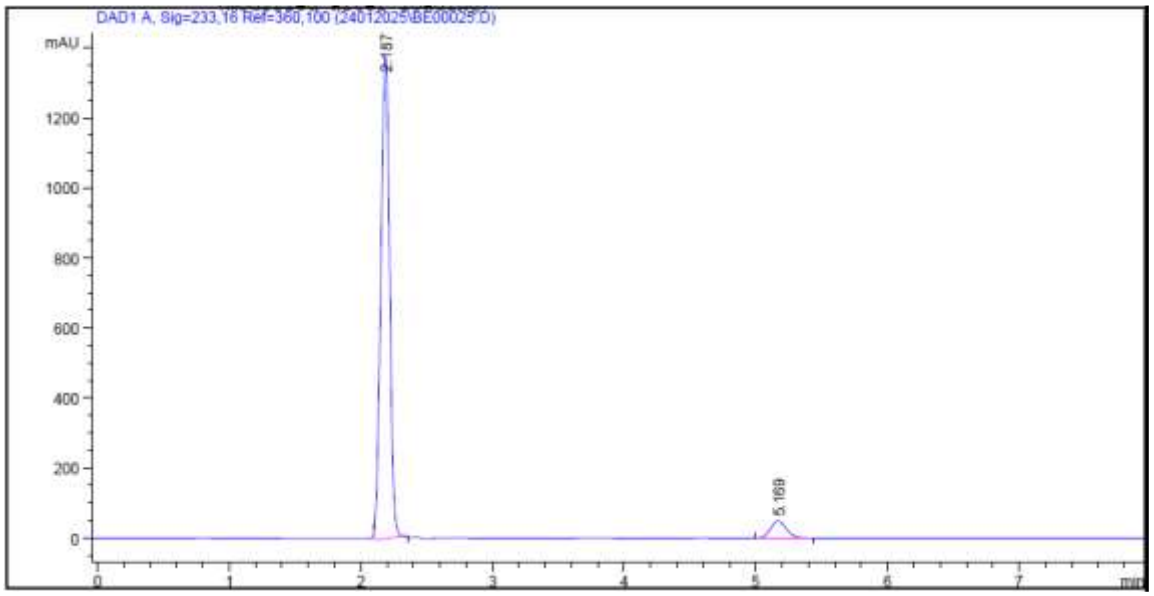


Fig No .47. Chromatogram of Mobile phase composition change 74ml Methanol+ 26ml 0.05% OPA

4) Mobile phase composition Change: 76ml Methanol+ 24ml 0.05% OPA

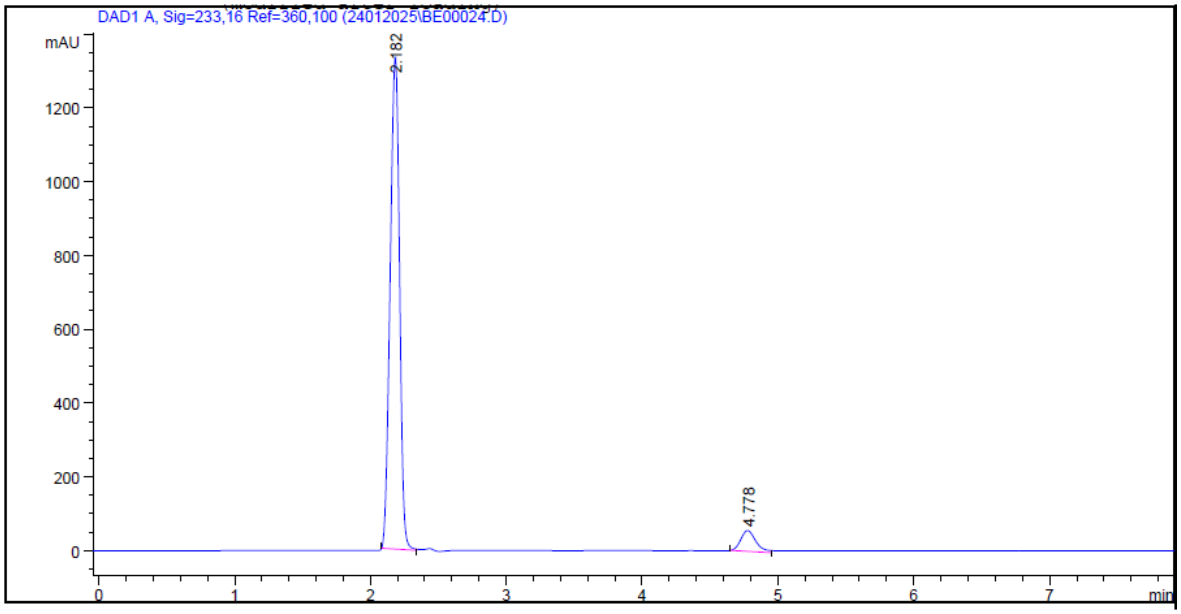


Fig No.48: Chromatogram of Mobile phase composition change 76ml Methanol+24 ml 0.05% buffer

5) Wavelength Change 232 nm

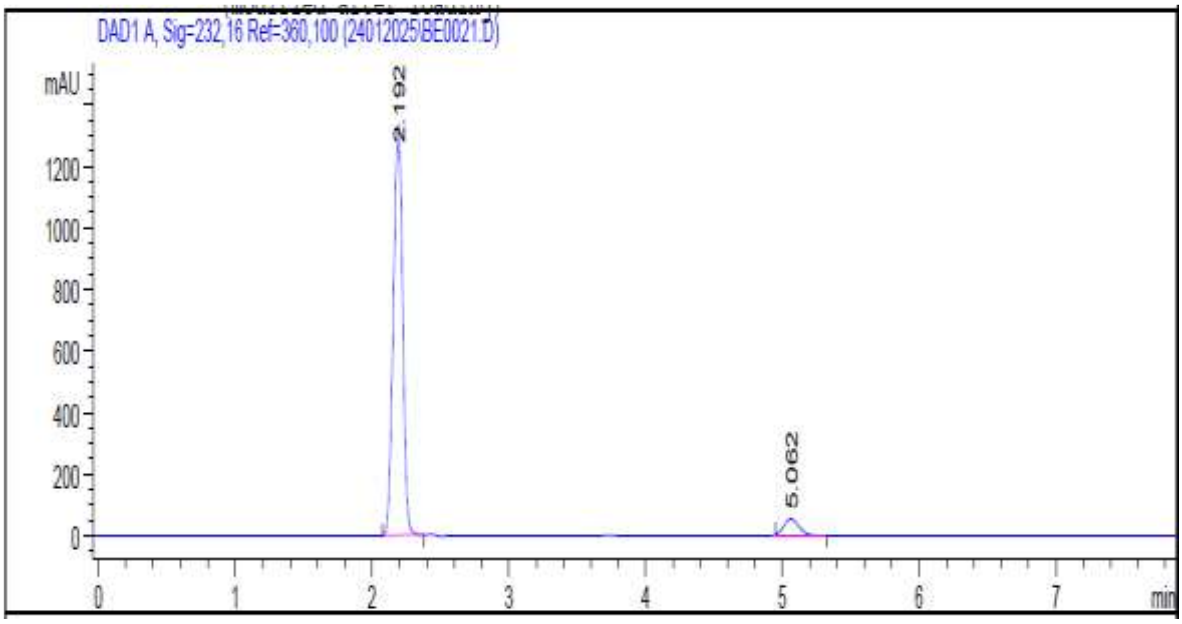


Fig.No 49: Chromatogram of comp change wavelength change 232 nm

6) Wavelength Change 234 nm

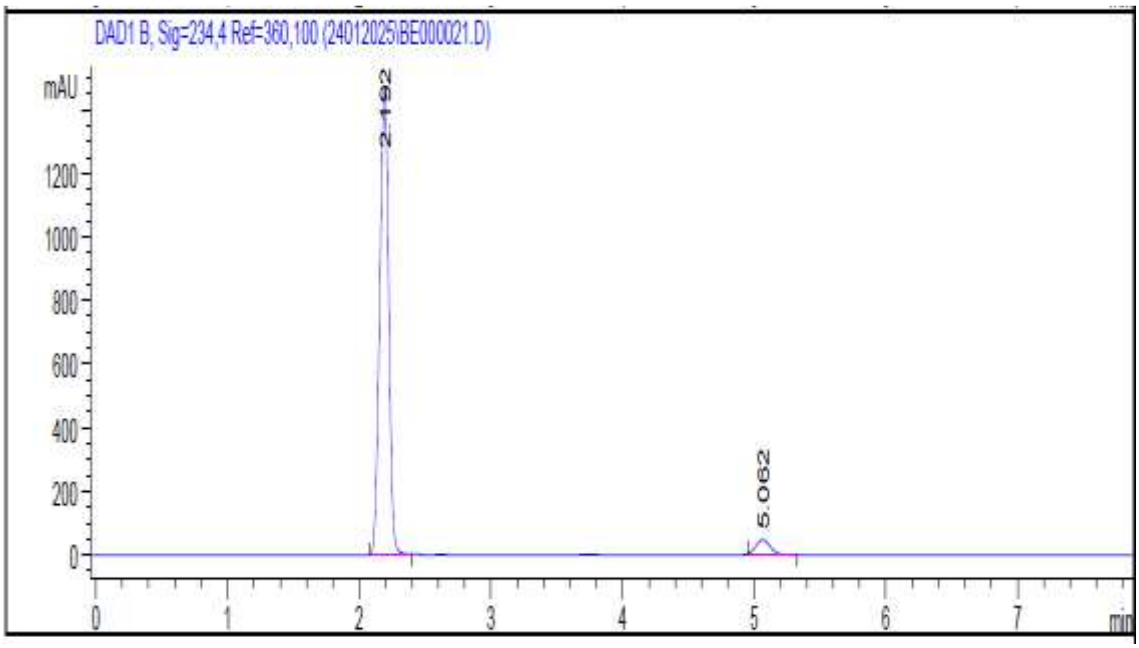


Fig.No 50: Chromatogram of comp change wavelength change 234nm

Table No.24 Result of Robustness Study of Bempedoic acid

Parameters	**Conc.(µg/ml)**	**Amount of detected(mean ±SD)**	**%RSD**
Chromatogram of flow change 0.9ml	72	7295.50±1.19	0.02
Chromatogram of flow change 1.1 ml	72	5958.91±15.04	0.25
Chromatogram of comp change 76ml Meoh+24ml OPA Water	72	6393.09±7.63	0.12
Chromatogram of comp change 74ml Methanol+26 ml OPA Water	72	6398.0±17.33	0.27
Chromatogram of comp change wavelength change 232nm	72	6308.4±7.30	0.12
Chromatogram of comp change wavelength change 234 nm	72	6954.02±12.58	0.18

Robustness Study of Bempedoic acid:

The changes were did flow rate (±1 ml/ min^{-1}),PH of mobile phase composition (±1 ml/ min^{-1}),and Wavelength (±1 ml/ min^{-1}) .%RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded.(**Table No.24)**

Table No.25. Result of Robustness Study of Ezetimibe

Parameters	**Conc.(µg/ml)**	**Amount of detected(mean ±SD)**	**%RSD**
Chromatogram of flow change 0.9ml	4	453.71±3.06	0.67
Chromatogram of flow change 1.1 ml	4	491.63±1.29	0.26
Chromatogram of comp change 76ml Meoh+24ml OPA Water	4	444.49±3.20	0.72
Chromatogram of comp change 74ml Methanol+26 ml OPA Water	4	404.7±0.97	0.24
Chromatogram of comp change wavelength change 232nm	4	429.3±1.37	0.32
Chromatogram of comp change wavelength change 234 nm	4	374.61±1.13	0.30

Robustness Study of Ezetimibe:

The changes were did flow rate (±1 ml/ min^{-1}),PH of mobile phase composition (±1 ml/ min^{-1}),and Wavelength (±1 ml/ min^{-1}) .%RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded**.(Table No.25)**

6. Limit Detection

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope The limit of detection (LOD) may be expressed as:

$$\mathbf{LOD = 3.3\ (SD)/S}$$

where, SD = Standard deviation of Y intercept
S = Slope

Limit of detection = 1.9876 (µg/mL)of Bempedoic acid
Limit of detection = 6.02 (µg/mL)of MTS

The LOD of Bempedoic acid was found to be 1.987 (µg/mL) and 6.02 (µg/mL), analytical method that concluded.

7. Limit Quantification

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope,
The quantitation limit (LOQ) may be expressed as:

LOQ = 10 (SD)/ S

where, SD = Standard deviation Y intercept
S = Slope

Limit of Quantitation = 0.060 (µg/mL)
Limit of Quantitation = 0.1845 (µg/mL)

The LOQ of Ezetimibe was found to be 0.060 µg/mL) and 0.1845 (µg/mL), analytical method that concluded.

5.3 Analysis of tablet formulation:-

Procedure:

Weigh 236mg Bempedoic acid and Ezetimibe combination tablet added into 50 ml volumetric flask. Add about 50 ml Methanol of diluent and sonicate to dissolve it and make volume up to the mark with diluent. Mix well and filter through 0.45 µm filter. Further pipette 0.4 ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents. (144 µg/ml and 8 µg/ml). The simple chromatogram of test Bempedoic acid and Ezetimibe Shown in **(Fig No: 51)** the amounts of Bempedoic acid and Ezetimibe were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated five times with tablet formulation. Tablet Assay for %Lable claim for %RSD Calculated, Result was shown in **(Table No. 26)**

Brand Name: Bempetol- EZ

1) Take 236 mg of formulation diluted with 50 ml methanol- Stock II
2) Take 0.4 ml in 10 ml Methanol from Stock II= 144 µgm/ml BPD and 8 µgm/ml ETM–II

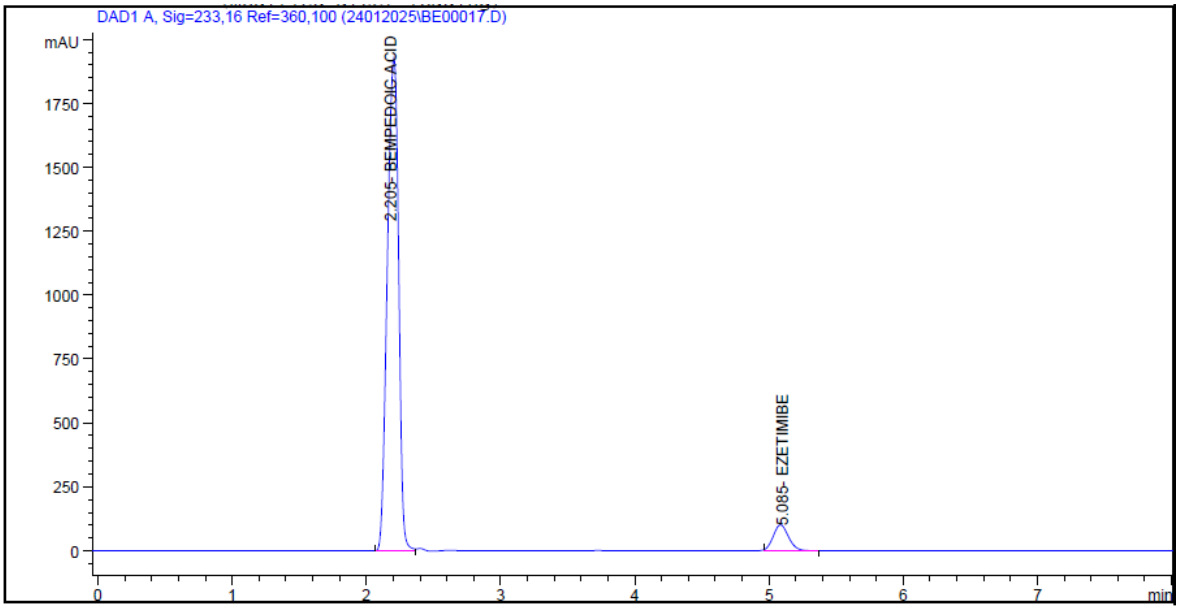


Fig No.51: Chromatogram for Marketed Formulation

Table.26. Analysis of marketed formulation.

Assay	Drug	Lable Claimed	Amt.Found	%Lable Claim	SD	%RSD
Rp-HPLC Method	**BPD**	144	143.349	99.55	0.042	0.029
	ETM	8	814.019	99.02	0.009	0.1155
	BPD	144	143.408	99.59	0.029	0.02
	ETM	8	815.68	99.10	0.116	0.116

Analysis of marketed formulation were also %Lable Claim was found to be 98-102% Satisfactory are concluded. (Table No.26)

6. Conclusion:

Simple, rapid, accurate and precise RP-HPLC methods have been developed and validated for the routine analysis of Bempedoic acid and Ezetimibe in API and tablet dosage forms. Both methods are suitable for the simultaneous determination of Bempedoic acid and Ezetimibe in multi-component formulations without interference of each other. The developed methods are recommended for routine and quality control analysis of the investigated drugs in two component pharmaceutical preparations. The amount found from the proposed methods was in good agreement with the label claim of the formulation. Also the value of standard deviation

and coefficient of variation calculated were satisfactorily low, indicating the suitability of the proposed methods for the routine estimation of tablet dosage forms.

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