

DEVELOPMENT AND VALIDATION OF A RP - HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF BEMPEDOIC ACID & EZETIMIBE IN PURE AND PHARMACEUTICAL DOSAGE FORM

S KRISHNA BHUVANAGIRI ¹ , JAYENDRA KUMAR ²

¹ Research Scholar, Associate Professor, A.K.R.G.College of Pharmacy, Nallajerla, 534112.

² Research Guide , Professor, School of Pharmacy, Monad University, Hapur, 245304.

Correspondence to Author:

**** S KRISHNA BHUVANAGIRI**

Associate Professor,
Department of Pharmaceutical Analysis,
A.K.R.G.College of Pharmacy,
Nallajerla,534112.

ABSTRACT:

Analytical Method Development and Validation for Bempedoic acid and Ezetimibe in bulk and Combined Dosage Form by RP-HPLC. Include Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Bempedoic acid and Ezetimibe in Bulk and their Pharmaceutical dosage form. Using Waters alliance HPLC system, Quaternary gradient pump of e2695 series equipped with an auto sampler injector with 10µl is injected eluted with the mobile phase containing HSA and Acetonitrile in the ratio of 30:70 v/v which is pumped at a flow rate of 1ml/min and detected by UV detector at 225nm. The peak of Bempedoic acid and Ezetimibe was eluted at retention times of 3.246 min and 3.865 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 and Ezetimibe, Method Development, Validation, Accuracy, Precision.

INTRODUCTION:

Bempedoic acid is indicated as an adjunct to diet and maximally tolerated statin therapy for adults with heterozygous familial hypercholesterolemia or existing atherosclerotic cardiovascular disease that warrants additional lowering of LDL-C. The combination of bempedoic and ezetimibe is also indicated with diet management and maximally tolerated statin therapy to treat elevated LDL-C levels in adults with heterozygous familial hypercholesterolemia or existing atherosclerotic cardiovascular disease who require further lowering of LDL-C [1-2]. IUPAC name is 8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid. Molecular Formula is C₁₉H₃₆O₅. Molecular weight is 344.4. Bempedoic acid is sparingly soluble in aqueous buffers. For maximum solubility in aqueous buffers, Bempedoic acid should first be dissolved in DMSO and then diluted with the aqueous buffer of choice.

Bempedoic acid has a solubility of approximately 0.25 mg/ml in a 1:3 solution of DMSO: PBS (pH 7.2) using this method.

Ezetimibe is indicated to reduce elevated total-C, LDL-C, Apo B, and non-HDL-C in patients with primary hyperlipidemia, alone or in combination with an HMG-CoA reductase inhibitor (statin). It is also indicated to reduce elevated total-C, LDL-C, Apo B, and non-HDL-C in patients with mixed hyperlipidemia in combination with fenofibrate, and to reduce elevated total-C and LDL-C in patients with homozygous familial hypercholesterolemia (HoFH), in combination with atorvastatin or simvastatin [3]. IUPAC name is (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidin-2-one. Molecular Formula is $C_{24}H_{21}F_2NO_3$. Molecular weight is 409.4. It dissolves very well in all kinds of organic solvents, e.g., ethanol, DMSO, DMF, but it is practically insoluble in water.

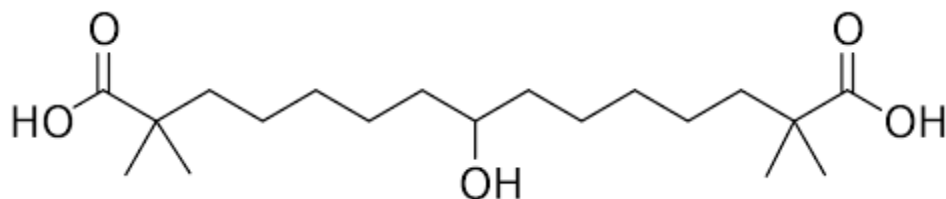


Figure 1: Structure of Bempedoic acid

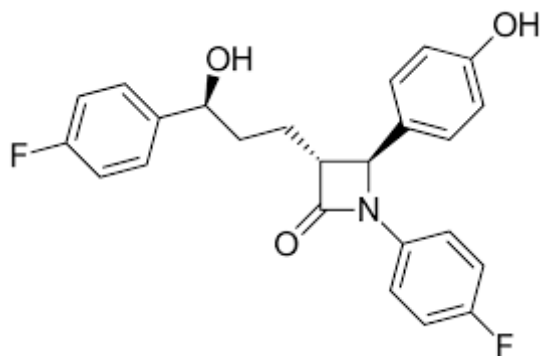


Figure 2: Structure of Ezetimibe

A literature survey conveyed that, limited methods are available for simultaneous estimation of Bempedoic acid and Ezetimibe. A few articles reported spectrophotometric techniques for estimation of Ezetimibe alone and with other drugs [4,5,6,7,8,9,10,11,12,13,14,15]. Few HPLC methods were reported for the determination of Ezetimibe alone and in combination with other drugs [16,17,18,19,20]. One RP-HPLC method was reported for simultaneous estimation of Bempedoic acid Ezetimibe [21]. Few LC-MS methods were reported for determination of Ezetimibe alone and in combination with other

drugs [22,23,24,25]. One LC–MS method was reported for estimation of Bempedoic acid in human plasma and urine [26]. In view of the demand for an appropriate, cost-effective RP-HPLC method for routine analysis of Bempedoic acid and Ezetimibe synchronized evaluation of in pharmaceutical dose type. Attempts were made to establish easy, precise, accurate as well as cost-efficient logical method for the estimate of Bempedoic acid and Ezetimibe.

The recommended approach will be validated according to ICH guidelines. The objective of the recommended work is to establish a brand-new, simple, delicate, exact and economical logical method as well as recognition for the Synchronized evaluation of Bempedoic acid and Ezetimibe in pharmaceutical dose kind by utilizing RP-HPLC. To verify the established method based on ICH standards for the desired analytical application.

MATERIALS AND METHODS:

Chemicals and Reagents: Bempedoic acid and Ezetimibe were purchased from Honour Lab. NaH_2PO_4 was analytical grade supplied by Finerchem limited, Orthophosphoric acid (Merck), and Water and Methanol for HPLC (Lichrosolv (Merck)).

Preparation of Mobile Phase: Mobile phase was prepared by mixing HSA and ACN taken in the ratio 30:70. It was filtered through a 0.45μ membrane filter to remove the impurities which may interfere in the final chromatogram.

Determination of Working Wavelength (λ_{max}):

In simultaneous estimation of two drugs isobestic wavelength was used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are inter convertible. So, this wavelength was used in simultaneous estimation to estimate two drugs accurately. The wavelength of maximum absorption of the solution of the drugs in mixture of Acetonitrile and HSA (70:30) were scanned using PDA Detector within the wavelength region of 200–400 nm against Acetonitrile and HSA (70:30) as blank. The absorption curve shows isobestic point at 225 nm. Thus 225 nm was selected as detector wavelength for the HPLC chromatographic method.

Chromatographic conditions:

During the selection of chromatographic conditions, numbers of trials were carried out and the best trial was selected for optimized method.

Preparation of standard stock solution

Accurately weigh and transfer 180 mg of Bempedoic acid, 10 mg of Ezetimibe working standard into a 100 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent. (18ppm of Bempedoic acid, 10ppm of Ezetimibe)

Sample Solution Preparation:

Accurately weighed and transfer 248mg of Bempedoic acid and Ezetimibe sample into a 100mL clean dry volumetric flask add Diluent and sonicate it up to 30 mins to dissolve, and centrifuge for 30min. to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45-micron Injection filter. (Stock solution). Further pipette 5 ml of the above stock solutions into a 50ml volumetric flask and dilute up to the mark with diluents. (180ppm of Bempedoic acid, 10ppm of Ezetimibe).

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

RESULTS AND DISCUSSION

METHOD:

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness and robustness as per ICH guidelines.

System suitability parameters: To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1.0 ml/min to equilibrate the column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 10 μ L of standard into Inertsil ODS (150x4.6 mm, 3.5 μ), the mobile phase of composition Acetonitrile: HSA (70:30) was allowed to flow through the column at a flow rate of 1.0 ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in table 1.

Table 1: System suitability parameters

S.no	Parameter	Bempedoic acid	Ezetimibe
1	Retention time	3.248	3.865
2	Plate count	4112	5004
3	Tailing factor	1.11	1.10
4	Resolution	----	2.92
5	%RSD	0.30	0.63

Assay of pharmaceutical formulation: The proposed validated method was successfully applied to determine Bempedoic acid and Ezetimibe in their pharmaceutical dosage form. The result obtained for was comparable with the corresponding labeled amounts and they were shown in Table-2.

Table 2: Assay results for Bempedoic acid and Ezetimibe

Brand	Drug	Avg sample area (n=5)	Std. wt (µg/ml)	Sample wt. (µg/ml)	Label amount (mg)	Std purity	Amount found (µg/ml)	% assay
NEXL IZET	Bempedoic Acid	4618965	180	248	180	99.8	179.27	99.6
	Ezetimibe	232365	10	248	10	99.9	10.02	100.2

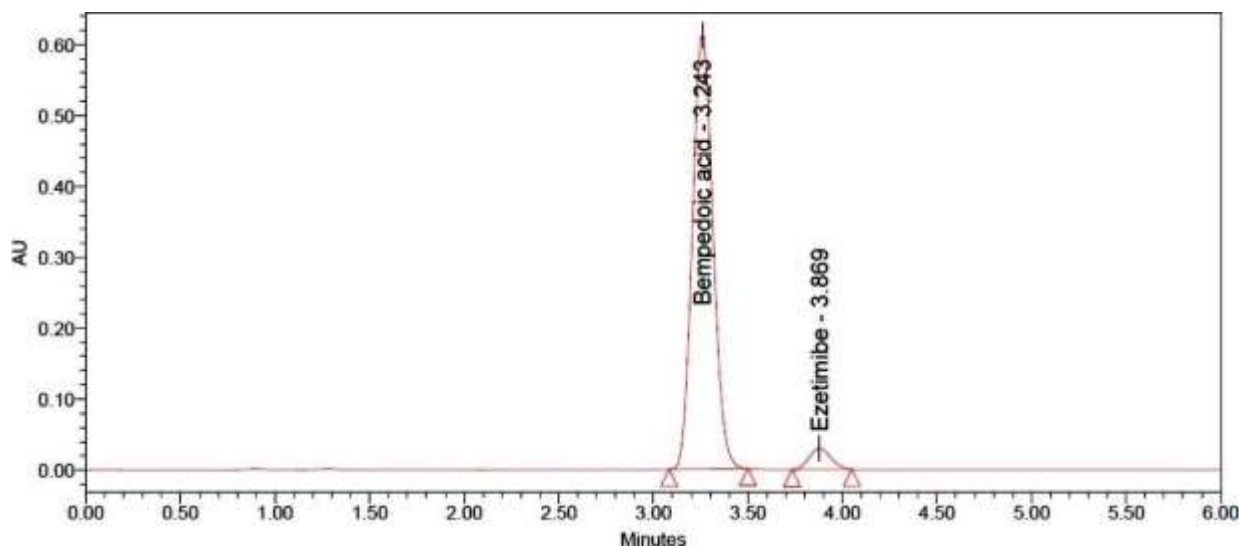


Figure 3: Standard chromatogram

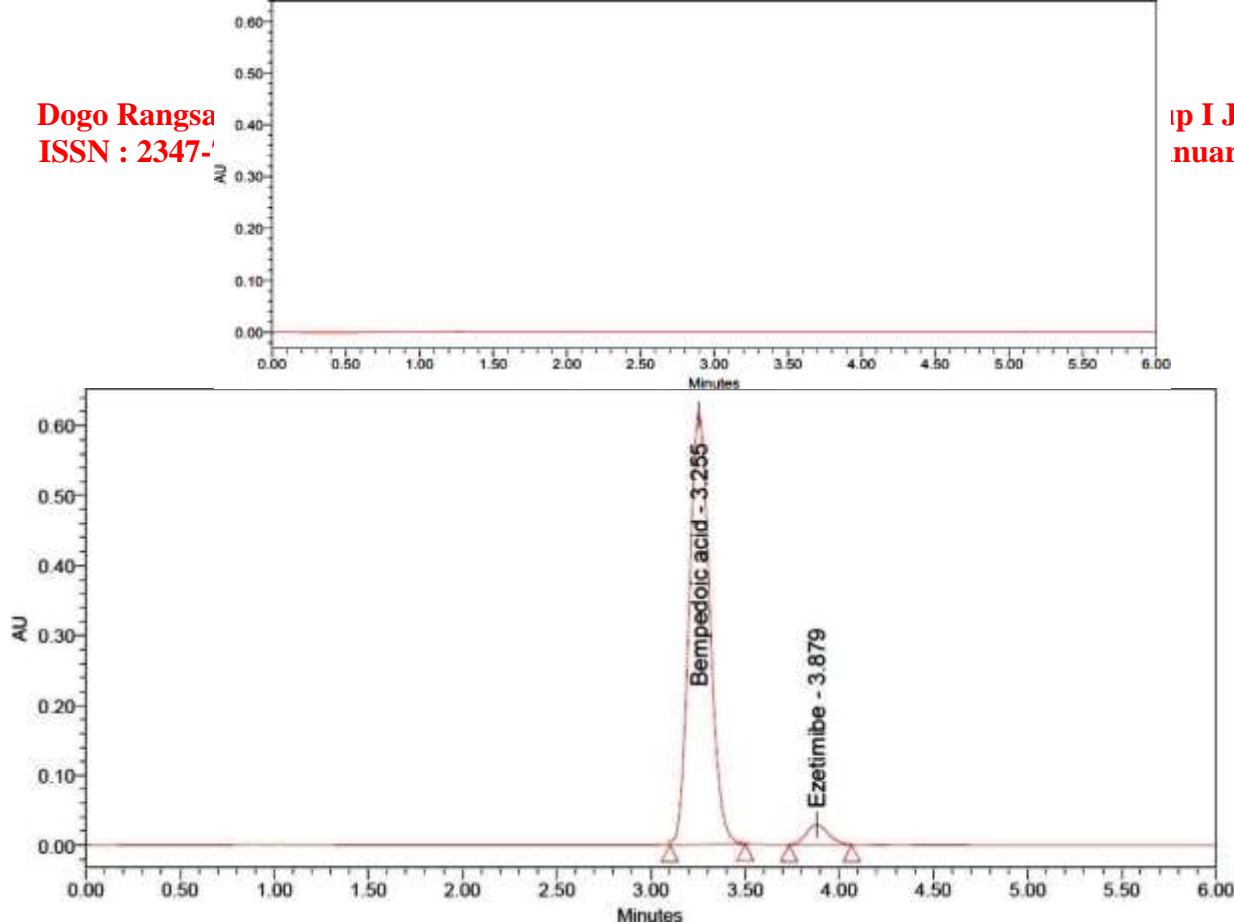


Figure 4: Sample chromatogram

Validation of Analytical method:

Linearity: The linearity study was performed for the concentration of 45 µg/ml to 270 µg/ml and 2.5 µg/ml to 15 µg/ml level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. 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. The results are shown in table 3.

Table 3: Results of linearity for Bempedoic acid & Ezetimibe

S.NO	Bempedoic acid		Ezetimibe	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	45.00	1329639	2.50	69046

2	90.00	2359735	5.00	120773
3	135.00	3394721	7.50	172648
4	180.00	4632381	10.00	230091
5	225.00	5729283	12.50	286974
6	270.00	7064333	15.00	351696
Regression equation	$y=25607.09x+44484.61$		$y=22860.89x+4433.07$	
Slope	25607.09		22860.89	
Intercept	44484.61		4433.07	
R²	0.9994		0.9993	

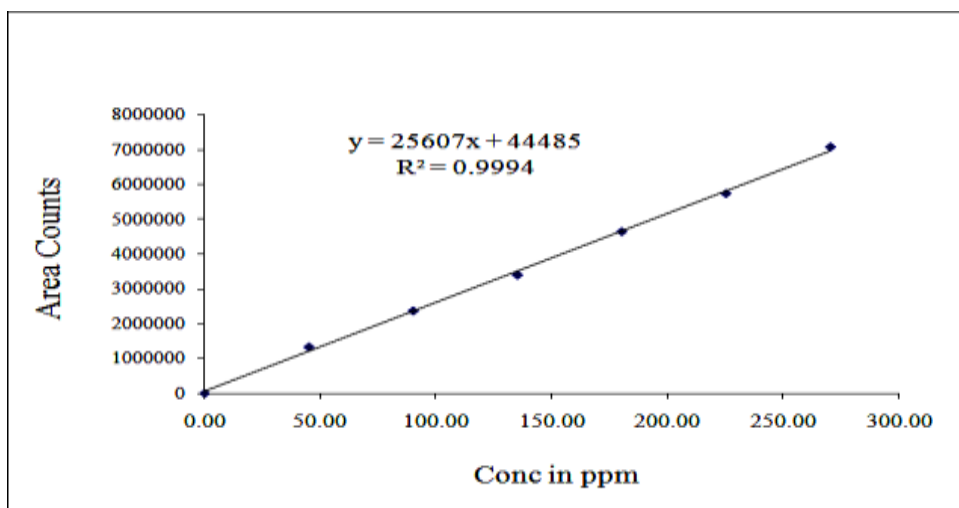


Figure 6: Linearity graph for Bempedoic acid

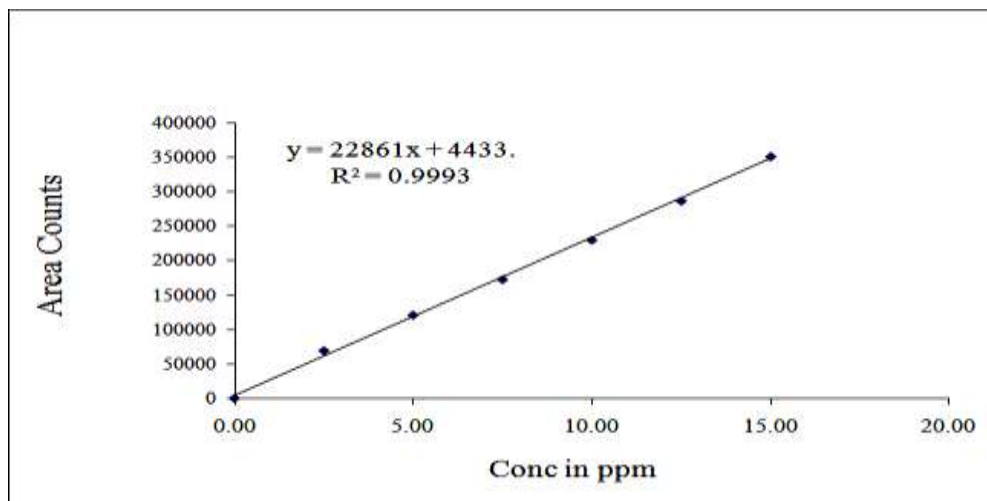


Figure 6: Linearity graph for Ezetimibe

Accuracy studies: The accuracy was determined by help of recovery study. The recovery method carried out at three level 50%, 100%, 150% and 50%, 100%, 150% Inject the standard solutions into chromatographic system. Calculate the Amount found and Amount added for Bempedoic acid and Ezetimibe and calculate the individual recovery and mean recovery values. The results are shown in table 4,5.

Table 4: Showing accuracy results for Bempedoic acid

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	%Recovery	Mean Recovery
50%	2309857	90	89.65	99.6	100.4
100%	4671441	180	181.31	100.7	
150%	7010608	270	272.10	100.8	

Table 5: Showing accuracy results for Ezetimibe

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	%Recovery	Mean Recovery
50%	117176	5	5.05	101.0	101.2
100%	236167	10	10.18	101.8	
150%	350163	15	15.1	100.7	

Precision Studies: precision was calculated from Coefficient of variance for six replicate injections of the standard. The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found. The results are shown in table 6.

Table 6: Precision results for Bempedoic acid and Ezetimibe

S.No	Concentration Bempedoic acid($\mu\text{g/ml}$)	Area of Bempedoic acid	Concentration of Ezetimibe($\mu\text{g/ml}$)	Area of Ezetimibe
1.	180	4648402	10	230043
2.	180	4629073	10	233617
3.	180	4635956	10	230667
4.	180	4659647	10	231479
5.	180	4631617	10	233308
6.	180	4621456	10	232627
Mean	4637692		231957	
S.D	13960.95		1455.04	
%RSD	0.30		0.63	

Ruggedness: To evaluate the intermediate precision of the method, Precision was performed on different day. The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found. The results are shown in table 7.

Table7: Ruggedness results of Bempedoic acid and Ezetimibe

S.No.	Area for Bempedoic acid		Area for Ezetimibe	
	Day-1	Day-2	Day-1	Day-2
1	4652261	4634652	234388	232686
2	4699950	4640724	232178	235978
3	4591334	4656481	234878	234629
4	4606891	4671708	232064	231547
5	4618068	4664553	235394	233265
6	4706522	4628639	234768	235903

Average	4645837	4649459	233945	234001
Standard Deviation	48807.047	17327.292	1449.422	1800.753
%RSD	1.05	0.37	0.62	0.77

Robustness: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. The flow rate was varied at 0.8 ml/min to 1.2ml/min. The results are shown in table 8,9.

Table 8: Robustness results of Bempedoic acid by RP-HPLC

Parameter	Bempedoic acid					
	Condition	Retention time(min)	Peak area	Resolution	Tailing	Plate count
Flow rate Change (mL/min)	Less flow (0.8 ml)	3.897	5291586		1.16	4138
	Actual (1ml)	3.246	4648402		1.11	4112
	More flow (1.2 ml)	2.806	4086390		1.08	4103
Organic Phase change	Less Org (63:37)	4.269	4809598		1.14	4166
	Actual (70:30)	3.245	4629073		1.10	4120
	More Org (77:23)	2.613	4367453		1.06	4102

Table 9: Robustness results of Ezetimibe by RP-HPLC

Parameter	Ezetimibe					
	Condition	Retention time(min)	Peak area	Resolution	Tailing	Plate count
Flow rate	Less flow	4.647	278468	3.34	1.13	5163

Change(mL/ min)	(0.8ml)					
	Actual(1ml)	3.865	230043	2.90	1.10	500 4
	More flow(1. 2ml)	3.340	206116	2.67	1.06	498 9
Organic Phasecha nge	Less Org(63 :37)	5.006	251565	3.15	1.14	505 8
	Actua l(70:3 0)	3.863	233617	2.93	1.09	500 7
	More Org(77: 23)	3.131	213043	2.68	1.04	498 6

LOD and LOQ: The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines. The results are shown in table 10.

$$\text{LOD} = 3.3\sigma/S \text{ and}$$

$$\text{LOQ} = 10 \sigma/S, \text{ where}$$

σ = Standard deviation of y intercept of regression line,

S = Slope of the calibration curve

Table 10: LOD, LOQ of Bempedoic acid and Ezetimibe

Nameofdrug	LOD($\mu\text{g/ml}$)	LOQ($\mu\text{g/ml}$)
Bempedoicacid	5.4	18
Ezetimibe	0.3	1

DEGRADATION STUDIES:

Preparation of stock: Accurately weigh and transfer 180mg of Bempedoic acid, 10mg of Ezetimibe working standard into a 100 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Acid degradation: Pipette 5 ml of above solution into a 50ml volumetric flask and 3 ml of 1N Hcl was added. Then, the volumetric flask was kept at 60°C for 6 hours and then neutralized with 1 N NaOH and make up to 50ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Alkali degradation: Pipette 5 ml of above solution into a 50ml volumetric flask and add 3ml of 1N NaOH was added. Then, the volumetric flask was kept at 60°C for 6 hours and then neutralized with 1N Hcl and make up to 50ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Peroxide degradation: Pipette 5 ml above stock solution into a 50ml volumetric flask, 1 ml of 3% w/v of hydrogen peroxide added in 50 ml of volumetric flask and the volume was made up to the

mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Reduction degradation: Pipette 5ml of Stock solution transferred into 50ml volumetric flask to this add 1ml of 10% Sodium Bisulphate and kept on bench top for 10min then the remaining procedure is same as the test preparation.

Thermal induced degradation: Bempedoic acid and Ezetimibe samples were taken in Petridish and kept in Hot air oven at 1100 C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Table 14: Forced Degradation results for Bempedoic acid and Ezetimibe

Results: %Degradati onresults	Bempedoicacid		Ezetimibe	
	Area	% Degradation	Area	%Degradation
Control	4639135	0	231891	0
Acid	3957265	14.7	201097	13.3
Alkali	3939838	15.1	196199	15.4
Peroxide	4074515	12.2	206507	10.9
Reduction	4149295	10.6	202407	12.7
Thermal	3997236	13.9	205271	11.5

CONCLUSION:

The Developed HPLC method was validated and it was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Bempedoic acid and Ezetimibe in its pure and pharmaceutical dosage form. Hence, this method can easily and conveniently adopt for routine quality control analysis of Ezetimibe and Bempedoic acid in its pure and pharmaceutical dosage form.

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