RP- HPLC Analytical Method Development and Validation for the Simultaneous Estimation of Bempedoic Acid and Ezetimibe in Bulk and Tablet Dosage Form

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ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Bempedoic acid and Ezetimibe in bulk and tablet dosage form.[1] Chromatogram was run through Kromosil C18 150 x 4.6 mm, 3.0µ. Mobile phase containing 0.01N KH_2PO_4 Buffer: Methanol taken in the ratio 70:30 was pumped through the column at a flow rate of 0.9ml/min. The buffer used in this method was 0.01N KH_2PO_4 buffer. Temperature was maintained at 30°C. The optimized wavelength selected was 260.0 nm. The retention time of Bempedoic acid and Ezetimibe was found to be 2.780min and 2.123min. %RSD of the Bempedoic acid and Ezetimibe were found to be 0.6 and 1.3 respectively. % Recovery was obtained as 99.87% and 100.12% for Bempedoic acid and Ezetimibe respectively. LOQ, and LOD values obtained from regression equations of Bempedoic acid and Ezetimibe were 0.10, 0.35, and 0.01, 0.03 respectively. The regression equation of Bempedoic acid is y = 28262x + 4418.6, and y = 28796x + 190.13 of Ezetimibe. Retention times were decreased and that run time was decreased, so the method developed was simple and economical and can be adopted in regular Quality control tests in Industries.

Keywords: Bempedoic acid, Ezetimibe, RP-HPLC

INTRODUCTION

Liquid chromatograph is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase to differing degrees due to differences in adsorption, ion exchange, partitioning, or size. These differences will allow the mixture components to be separated from each other by using these differences to determine the time of the solutes through a column. During this time pressure liquid chromatography began to be used to decrease flow through time, thus reducing the separation time of compounds being isolated by column chromatography. However, flow rates were inconsistent, and the question of whether it was better to have a constant flow rate or constant pressure was debated. High-pressure liquid chromatography quickly improved with the development of column packing materials. Additional convenience of online detectors became rapidly a powerful separation technique and is today called High-Performance Liquid Chromatography (HPLC).[2]

Bempedoic Acid:

High levels of LDL cholesterol (LDL-C) are a major risk factor for cardiovascular events. Caused by genetic mutations or lifestyle factors, hypercholesterolemia can significantly reduce quality of life and increase the risk of mortality from cardiovascular disease.9 About 1 in 4 patients, or 15 million Americans with elevated LDL-C, are insufficiently managed with maximally tolerated statin therapy alone, requiring additional treatment for hypercholesterolemia.[3] Bempedoic acid is first-in-class adenosine triphosphate-citrate lyase (ACL) inhibitor used once a day for reducing LDL cholesterol levels in statin-refractory patients. It was developed by Esperion Therapeutics Inc. and approved by the FDA on February 21, 2020. A combination product of bempedoic acid and ezetimibe was approved on February 26, 2020 for increased control of LDL cholesterol levels in patients experiencing refractory elevations despite previous statin treatment.

Ezetimibe:

Ezetimibe is a lipid-lowering compound that inhibits intestinal cholesterol and phytosterol absorption. The discovery and research of this drug began in the early 1990s, after the intravenous administration of radiolabelled ezetimibe in rats revealed that it was being localized within enterocytes of the intestinal villi - this prompted studies investigating the effect of ezetimibe on intestinal cholesterol absorption.3 Ezetimibe is used as an adjunctive therapy to a healthy diet to lower cholesterol levels in primary hyperlipidemia, mixed hyperlipidemia, homozygous familial hypercholesterolemia (HoFH), and homozygous sitosterolemia (phytosterolemia).Unlike other classes of cholesterol-reducing compounds including statins and bile acid sequestrants, ezetimibe

has a distinct mechanism of action involving the sterol transporter Niemann-Pick C1-Like 1 (NPC1L1), and is unique in that it does not affect the absorption of fat-soluble nutrients such as fat-soluble vitamins, triglycerides, or bile acids.4 In genetically NPC1L1-deficient mice, a 70% reduction in intestinal cholesterol absorption was seen, and these mice were insensitive to ezetimibe treatment - it was determined based on these findings that NPC1L1 plays an essential role in promoting intestinal cholesterol uptake via an ezetimibe-sensitive pathway.[4].3 By interfering with the intestinal uptake of cholesterol and phytosterols, ezetimibe reduces the delivery of intestinal cholesterol to the liver.

MATERIALS AND METHOD

Materials and reagents

Ezetimibe and Bempedoic acid pure drugs (API), Combination Ezetimibe and Bempedoic acid tablets (Bempesta-EZ_ Ezetimibe 180mg, Bempedoic Acid 10mg), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dehydrogenate ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem[5]

Instrumentation:

S.No	Instrument	Model
1	HPLC	Waters HPLC 2695 SYSTEM, software: Empower 2 software, PDA detector
2	UV/VIS Spectrophotometer	UV win 6 software
3	pH meter	BVK enterprises, India
4	Electronic balance	Dever
5	Ultrasonicator	BVK enterprises
6	Beakers	Borosil

Preparation of Standard stock solutions:

Accurately weighed 2.5mg of Ezetimibe, and 45mg of Bempedoic acid and transferred to a 50ml volumetric flask. 3/4th of diluents was added to both of these flasks and sonicated for 10 minutes. Flasks were made up of diluents and labeled as Standard stock solutions 1 and 2. (50µg/ml of Ezetimibe and 900µg/ml of bempedoic acid)

Preparation of Standard working solutions (100% solution):

1ml from stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (5µg/ml Ezetimibe of and 90µg/ml of Bempedoic acid) [6]

Preparation of Sample stock solutions:

5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 10ml volumetric flask, and 5ml of diluents were added

and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (100µg/ml of Ezetimibe and 1800µg/ml of bempedoic acid)

Preparation of Sample working solutions (100% solution):

0.5ml of filtered sample stock solution was transferred to a 10ml volumetric flask and made up with diluent. (5µg/ml of Ezetimibe and µg/ml of bempedoic acid)

VALIDATION OF METHOD DEVELOPED:

System suitability parameters:

The system suitability parameters were determined by preparing standard solutions of Ezetimibe (5ppm) and Bempedoic acid (90ppm) the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. [7] The % RSD for the area of six standard injections results should not be more than 2%.

Specificity:

Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision:

The repeatability of the method was verified by calculating the % RSD of six replicate injections of 100% concentration (60µg/ml of bempedoic and 40µg/ml of ezetimibe) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. [8]

Linearity:

By appropriate aliquots of the standard bempedoic and ezetimibe prepared six working solutions ranging between 15-90µg/mL& 10-60µg/ml. Each experiment linearity point was performed in triplicate according to optimized chromatographic conditions. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficient on curves for bempedoic and ezetimibe.

Accuracy:

Accuracy was carried out by % recovery studies of bempedoic and ezetimibe at three different concentration levels (50%, 100%, and 150%). Percentage recovery was calculated from the amount added and the amount recovered. The percentage recovery was within the acceptance criteria, this indicates the accuracy of the method [9]

Robustness:

Small deliberate changes in a method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guidelines.

Robustness conditions like Flow minus (0.8ml/min), Flow plus (1.0ml/min), mobile phase minus, mobile phase plus, temperature minus (23°C) and temperature plus(34°C) were maintained and samples were injected in a duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit. [10]

LOD sample Preparation:

0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.3ml each of Ezetimibe, and bempedoic acid, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents. [11]

LOQ sample Preparation:

0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluent. From the above solutions 0.9ml each of Ezetimibe, bempedoic acid, and solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent. [12]

Degradation studies:

Oxidation:

To 1 ml of stock solution of Ezetimibe and Bempedoic acid, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0c. For the HPLC study, the resultant solution was diluted to obtain 5μg/ml & and 90μg/ml solution and 10μl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Acid Degradation Studies:

To 1 ml of stock solution Ezetimibe and Bempedoic acid, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 5μg/ml & and 90μg/ml solution and 10μl solutions were injected into the system and the chromatograms were recorded to assess the stability of the sample. [13]

Alkali Degradation Studies:

To 1 ml of stock solution Ezetimibe and Bempedoic acid, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 5μg/ml & and 90μg/ml solution and 10μl were injected into the system and the chromatograms were recorded to assess the stability of the sample. [14]

Dry Heat Degradation Studies:

The standard drug solution was placed in the oven at 105°C for 6h to study dry heat degradation.

For the HPLC study, the resultant solution was diluted to 5µg/ml & and 90µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. [15]

RESULTS AND DISCUSSION:

System suitability:

All the system suitability parameters were within the range and satisfactory as per ICH guidelines.

Table: 1. System suitability parameters for Bempedoic acid and Ezetimibe

S no	Ezetimibe			Bempedoic acid			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.119	7255	1.46	2.773	9342	1.32	6.0
2	2.120	7071	1.45	2.774	9111	1.34	5.9
3	2.121	7101	1.43	2.774	9133	1.34	5.9
4	2.122	7068	1.39	2.776	9291	1.33	5.9
5	2.122	7058	1.39	2.779	9331	1.29	5.9
6	2.123	6825	1.38	2.780	9708	1.28	6.0

According to ICH guidelines plate count should be more than 2000, the Tailing factor should be less than 2 and the resolution must be more than 2 All the system-suitable parameters were passed and were within the limits.
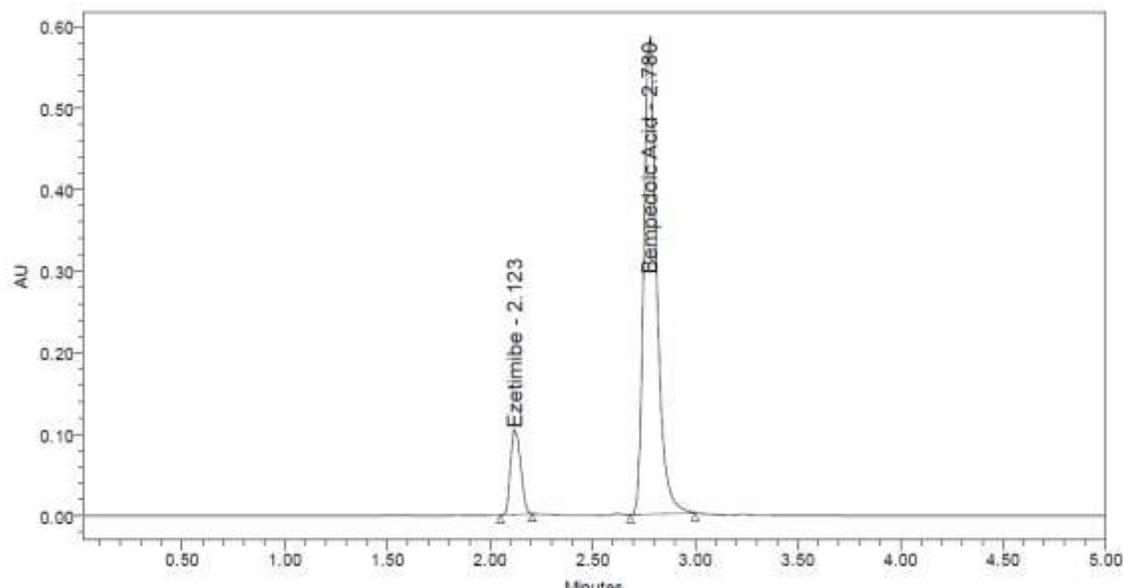

Figure 1: Optimized chromatogram

Retention times of Bempedoic acid and Ezetimibe were 2.780min and 2.123min respectively. We did not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity:

Table 2: Linearity table for Bempedoic acid and Ezetimibe.

Bempedoic acid		Ezetimibe	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
0	0	0	0
22.5	631669	1.25	36565
45	1260433	2.5	72349
67.5	1968558	3.75	108743
90	2533893	5	141965
112.5	3175555	6.25	181453
135	3814602	7.5	216160
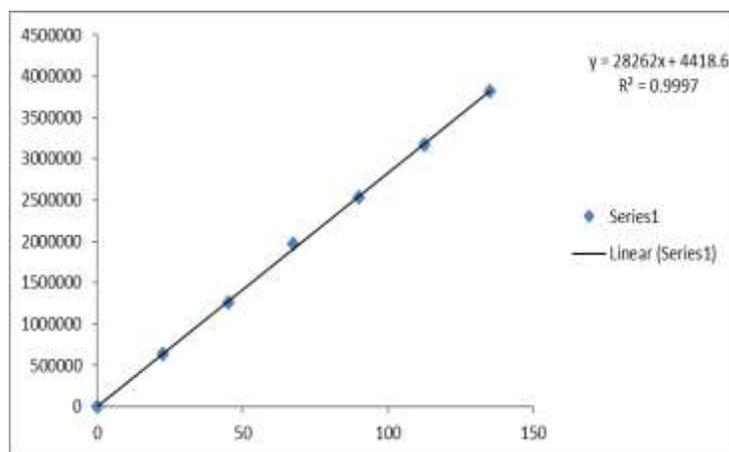
Figure 2: Calibration curve of Bempedoic acid
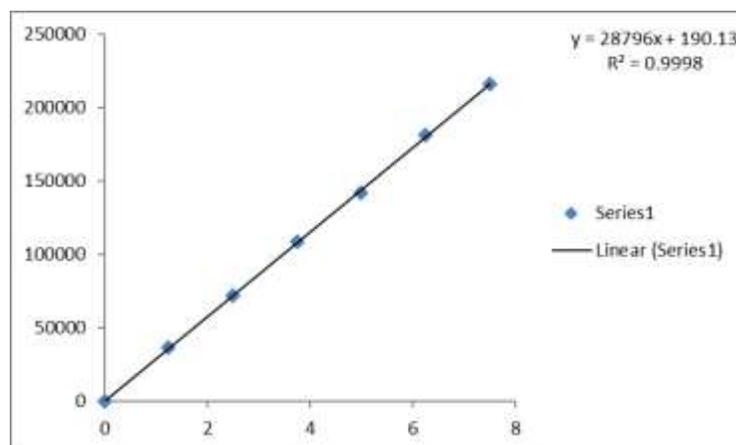
Figure 3: Calibration curve of Ezetimibe

Six linear concentrations of Bempedoic acid (22.5-135µg/ml) and Ezetimibe (1.25-7.5µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Bempedoic acid was y = 28262x + 4418.6 and for Ezetimibe was y = 28796x + 190.13. The correlation coefficient obtained was 0.9999 for the two drugs.

Precision:

Table 3: System precision table of Bempedoic acid and Ezetimibe

S. No	Area of Bempedoic acid	Area of Ezetimibe
1.	2549939	142248
2.	2567736	143725
3.	2556913	144732
4.	2543033	145677
5.	2524445	145470
6.	2564781	147877
Mean	2551141	144955
S.D	15966.3	1908.7
%RSD	0.6	1.3

From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.6% and 1.3% respectively for Bempedoic acid and Ezetimibe. As the limit of Precision was less than "2" the system precision was passed in this method.

Repeatability:

Table 4: Repeatability table of Bempedoic acid and Ezetimibe

S.No.	Area of Bempedoic acid	Area of Ezetimibe
1.	2544213	145434
2.	2555464	145949
3.	2565409	146426
4.	2572972	144525
5.	2567266	143947
6.	2558464	145449
Mean	2560631	145288
S.D	10199.0	912.1
%RSD	0.4	0.6

Multiple sampling from a sample stock solution was done and six working sample solutions of the same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.4% and 0.6% respectively for Bempedoic acid and Ezetimibe. As the limit of Precision was less than "2" the system precision was passed in this method

Accuracy:

Table 5: Accuracy table of Bempedoic acid

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean
50%	45	44.92	99.81	100.09
	45	45.42	100.93	
	45	44.85	99.68	
100%	90	90.12	100.13	99.61
	90	89.43	99.36	
	90	89.19	99.09	
150%	135	133.87	99.16	100.65
	135	135.10	100.07	
	135	135.74	100.55	

Table 6: Accuracy table of Ezetimibe

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean
50%	2.5	2.51	100.35	100.14
	2.5	2.48	99.05	
	2.5	2.52	100.88	
100%	5	4.99	99.84	99.5
	5	4.96	99.19	
	5	4.99	99.82	
150%	7.5	7.57	100.88	99.92
	7.5	7.54	100.52	
	7.5	7.54	100.56	

Three levels of Accuracy samples were prepared by the standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.87% and 100.12% for Bempedoic acid and Ezetimibe respectively.

Sensitivity:

Table 7: Sensitivity table of Bempedoic acid and Ezetimibe

Molecule	LOD	LOQ
Bempedoic acid	0.10	0.35
Ezetimibe	0.01	0.03
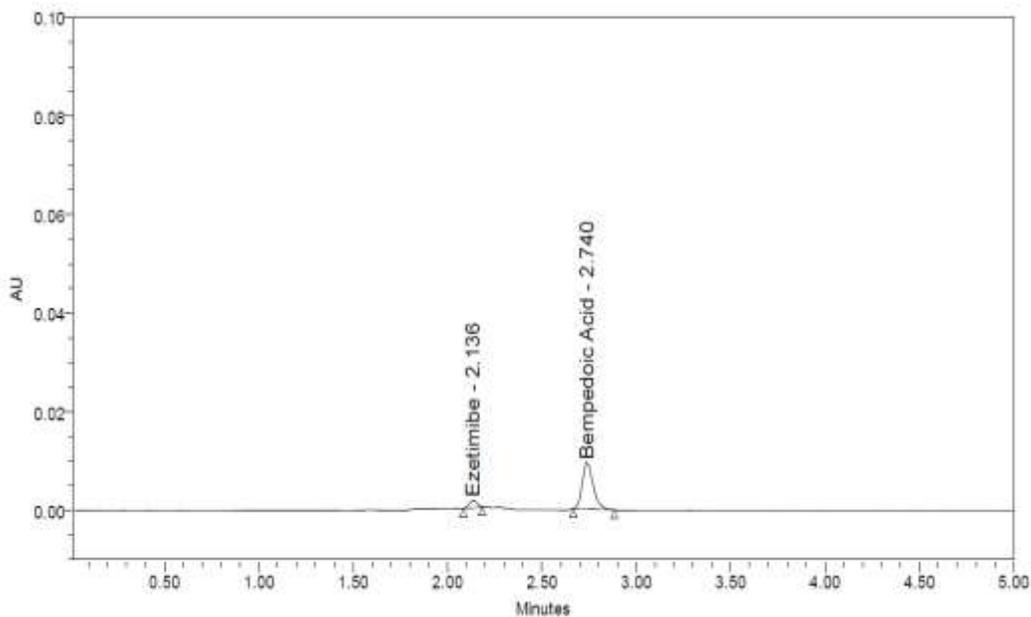
Figure 4: LOD Chromatogram of Standard
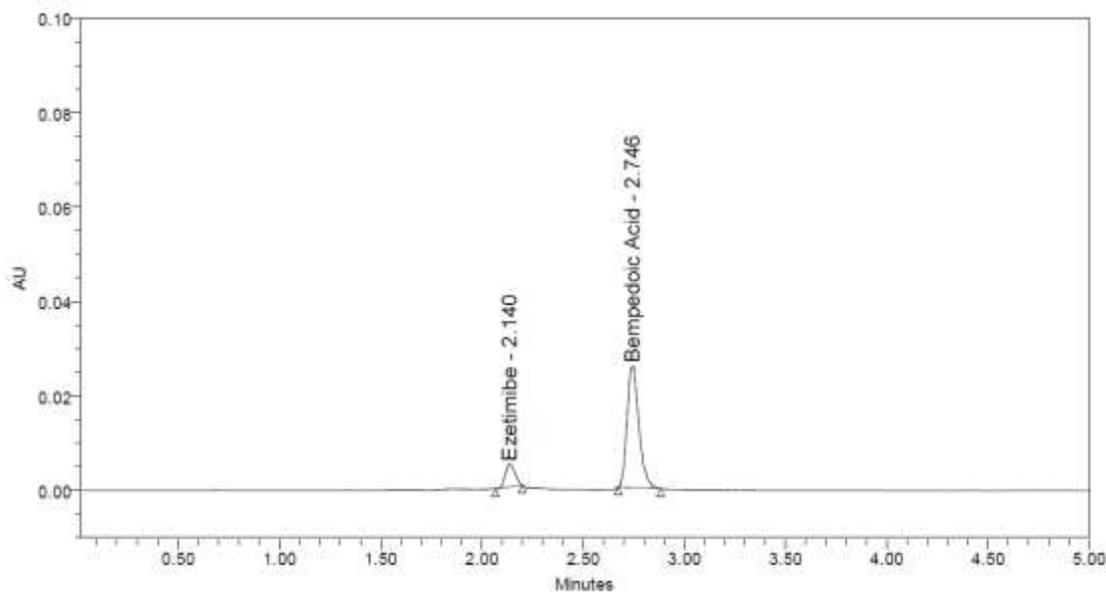
Figure 5: LOQ Chromatogram of Standard

Robustness:

Table 8: Robustness data for Bempedoic acid and Ezetimibe

S.no	Condition	%RSD of Bempedoic acid	%RSD of Ezetimibe
1	Flow rate (-) 0.8ml/min	0.4	1.6
2	Flow rate (+) 1.0ml/min	0.3	1
3	Mobile phase (-) 65B:35A	0.2	1.8
4	Mobile phase (+) 75B:25A	0.1	0.5
5	Temperature (-) 24°C	0.2	1.9
6	Temperature (+) 34°C	0.2	0.8

Robustness conditions like Flow minus (0.8ml/min), Flow plus (1.0ml/min), mobile phase minus (65B:35A), mobile phase plus (75B:25A), temperature minus (24°C) and temperature plus(34°C) was maintained and samples were injected in Triplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Assay:

Bempetol, bearing the label claim Bempedoic acid 180mg, Ezetimibe 10mg. Assay was performed with the above formulation. The average % Assay for Bempedoic acid and Ezetimibe obtained was 100.27 and 100.13% respectively.

Table 9: Assay Data of Bempedoic acid

S.no	Standard Area	Sample area	% Assay
1	2549939	2544213	99.63
2	2567736	2555464	100.07
3	2556913	2565409	100.46
4	2543033	2572972	100.75
5	2524445	2567266	100.53
6	2564781	2558464	100.19
Avg	2551141	2560631	100.27
Std ev	15966.3	10199.0	0.3994
%RSD	0.6	0.4	0.4

Table 10: Assay Data of Ezetimibe

S.no	Standard Area	Sample area	% Assay
1	142248	145434	100.23
2	143725	145949	100.59
3	144732	146426	100.91
4	145677	144525	99.60
5	145470	143947	99.21
6	147877	145449	100.24
Avg	144955	145288	100.13
Stdev	1908.7	912.1	0.63
%RSD	1.3	0.6	0.6

Degradation Data:

Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation.

Table 11: Degradation Data of Bempedoic acid

S.NO	Degradation Condition	% Drug Degraded	% drug undegraded
1	Acid	97.49	2.51
2	Alkali	93.40	6.60
3	Oxidation	93.51	6.49
4	Thermal	97.70	2.30
5	UV	98.38	1.62
6	Water	98.69	1.31

Table 12: Degradation Data of Ezetimibe

S.NO	Degradation Condition	% Drug Degraded	% drug undegraded
1	Acid	97.76	2.24
2	Alkali	93.10	6.90
3	Oxidation	93.60	6.40
4	Thermal	97.62	2.38
5	UV	98.89	1.11
6	Water	99.81	0.19

CONCLUSION:

A simple, Accurate, precise method was developed for the simultaneous estimation of the Bempedoic acid and Ezetimibe in Bulk and Pharmaceutical dosage forms. Retention time of Bempedoic acid and Ezetimibe was found to be 2.780min and 2.123min. %RSD of the Bempedoic acid and Ezetimibe were found to be 0.6 and 1.3 respectively. %Recovery was obtained as 99.87% and 100.12% for Bempedoic acid and Ezetimibe respectively. LOQ, and LOD values obtained from regression equations of Bempedoic acid and Ezetimibe were 0.10, 0.35 and 0.01, 0.03 respectively. The regression equation of Bempedoic acid is $y = 28262x + 4418.6$, and $y = 28796x + 190.13$ of Ezetimibe. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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