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Research Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF BEMPEDOIC ACID AND EZETIMIBE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT
New method was established for simultaneous estimation of Bempedoic acid and Ezetimibe by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Bempedoic acid and Ezetimibe by using ACE C18 column (4.6×150mm) 5μ, flow rate was 1.2 ml/min, mobile phase ratio was (70:30 v/v) methanol:Phosphate buffer pH 3 (pH was adjusted with orthophosphoricacid), detection wavelength was 240nm. The instrument used was Shimadzu, model No. SPD-20MA LC+20AD, Software- LC-20 Solution. The retention times were found to be 2.733 mins and 3.415 mins. The % purity of Bempedoic acid and Ezetimibe was found to be 101.27% and 99.97% respectively. The precision study was precise, robust, and repeatable. LOD value was 3.17 and 5.68, and LOQ value was 0.0172 and 0.2125 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Bempedoic acid and Ezetimibe in API and Pharmaceutical dosage form.

Keywords ACE C18 column, Bempedoic acid and Ezetimibe, RP-HPLC.

INTRODUCTION

High-performance liquid chromatography also known as High-pressure or High price or High-speed liquid chromatography, HPLC) is a form of column chromatography used frequently in analytical chemistry and biochemistry to identify, separate, and quantify compounds. It is a powerful tool in analysis. It is basically an improved form of column chromatography which has been optimized to provide rapid high-resolution separations. Early LC used gravity fed open tubular columns with particles 100s of microns in size; the human eye was used for a detector and separations often took hours or even days to develop. HPLC is probably the most universal type of analytical procedure. In addition, HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. Its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packings.

Reversed-phase chromatography uses a non-polar stationary phase and a polar mobile phase. This is the most common type of HPLC separation in use today. A partition mechanism is typically used for separations by non-polar differences. For reversed phase, alkyl hydrocarbons are the preferred stationary phase; octadecyl (C18) is the most common stationary phase, but octyl (C8) and butyl (C4) are also used in some applications. In reversed phase chromatography, the most polar compounds elute first with the most non-polar compounds eluting last.

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

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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. 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.6,7 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 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 radio labelled 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).

Chemical separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates for a given set of column and mobile phase. Purification refers to the process of separating or extracting the target compound from other (possibly structure related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic condition. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound. Identification of the compounds by HPLC is a crucial part of any HPLC assay. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from the extraneous peaks at the detection levels in which the assay would be performed. Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a solution. It involves injecting a series of known concentration of the standard compound solution onto the HPLC for detection. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected.

MATERIALS AND METHODS

Chemicals and standards used Water, Methanol, Acetonitrile, Ortho phosphoric acid, KH2PO4, K2PO4,0. 22µ Nylon filter,0.45µ filter paper, Tancodep-2, Bempedoic acid and Ezetimibe. Method development for the simultaneous estimation of Bempedoic acid and Ezetimibe by using RP-HPLC, Selection of mobile phase, Selection of detection wavelength, Selection of column, Selection of solvent delivery system, Selection of flow rate, Selection of column temperature, Selection of diluent, Selection of test concentration and injection volume. Chromatographic trials for simultaneous estimation of Bempedoic acid and Ezetimibe by RP- HPLC. The trial shows no proper separation peaks in the chromatogram, so more trials were required for obtaining peaks. Chromatogram showing trial-2 injection, in this trial two peaks were separated but don't have proper resolution. Still more trials were required for proper peaks. Chromatogram showing trial-3 injection, In this trial both Bempedoic acid and Ezetimibe were eluted but there is no proper resolution. Still more trials were required for better resolution in peaks. The separation was good, peak shape was good, so we conclude that there is no required for reduce the retention times of peaks, so it is taken as final method. Validation parameters are Specificity, Linearity, Range, Accuracy, Precision, Repeatability, Intermediate Precision, Detection Limit, Quantitation Limit, Robustness.

Assay Preparation of the Bempedoic acid and Ezetimibe standard and sample solution. Sample solution preparation:10 mg of Bempedoic acid and 1 mg Ezetimibe tablet powder were accurately weighed and transferred into a 10 ml clean dry volumetric flask, add about 2ml of diluent and sonicate to dissolve it completely and making volume up to the mark with the same solvent(Stock solution). Further pipette 10ml of the above stock solution into a 100ml volumetric flask and was diluted up to the mark with diluent. Standard solution preparation:10 mg Bempedoic acid and 1 mg Ezetimibe working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

Assay calculation

$$\text{Assay \%} = \frac{\text{sample area}}{\text{Standard area}} \times \frac{\text{dilution sample}}{\text{dilution of standard}} \times \frac{P}{100} \times \frac{\text{Avg. wt}}{Lc} \times 100$$

Where:

Avg.wt = average weight of tablets
P= Percentage purity of working standard
LC= Label Claim of Bempedoic acid   mg/ml.

Instruments used

Table 1: List of instruments used

S.No	Instrument name	Model number	Soft ware	Manufacturers Name
1	HPLC- Shimadzu	model No. SPD-20MA LC+20AD	Software- LC-20 Solution	Waters
2	U.V double beam spectrometer	UV 3000+	U.V win soft ware	Lab India
3	Digital weighing balance (sensitivity 5mg)	ER 200A	-	Ascoset
4	pH meter	AD 102U	-	ADWA
5	Sonicator	SE60US	-	Enertech

Method development for the simultaneous estimation of Bempedoic acid   and Ezetimibe by using RP-HPLC.
1. Selection of mobile phase
2. Selection of detection wavelength
3. Selection of column
4. Selection of solvent delivery system
5. Selection of flow rate
6. Selection of column temperature
7. Selection of diluent
8. Selection of test concentration and injection volume

Chromatographic trials for simultaneous estimation of Bempedoic acid and Ezetimibe by RP- HPLC

Trial-1

Chromatographic conditions

Column	:	Thermosil C18 4.6x150mm, 5µm
Mobile phase ratio	:	MeOH: H2O (60:40%v/v)
Detection wavelength	:	236nm
Flow rate	:	1ml/min
Injection volume	:	10µl
Column temperature	:	Ambient
Auto sampler temperature	:	Ambient
Run time	:	10min.

Figure 1: Chromatogram showing trial-1 injection
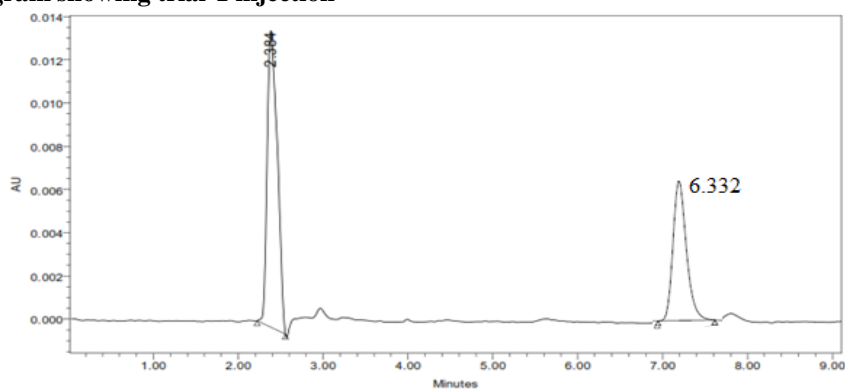
Observation:

The trial shows no proper separation peaks in the chromatogram, so more trials were required for obtaining peaks.

RESULTS AND DISCUSSION

The present investigation reported in the thesis was aimed to develop a new method development and validation for the simultaneous estimation of Bempedoic acid and Ezetimibe by RP-HPLC method. Literature reveals that there are no analytical methods reported for the simultaneous estimation Bempedoic acid and Ezetimibe by RP-HPLC method. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Bempedoic acid and Ezetimibe in pharmaceutical dosage form. The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual
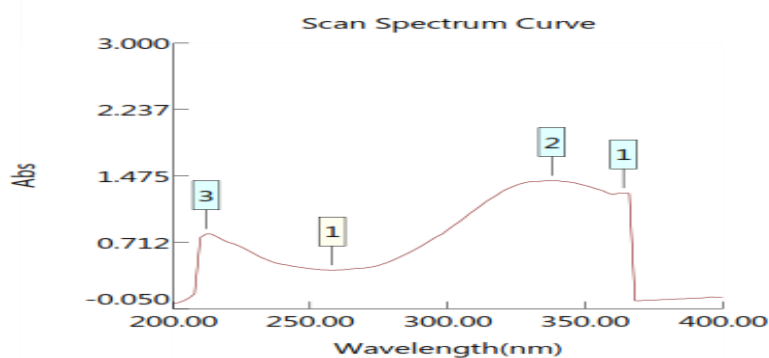
and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Bempedoic acid and Ezetimibe was obtained and the isobestic point of Bempedoic acid and Ezetimibe showed absorbance's maxima at 240 nm.
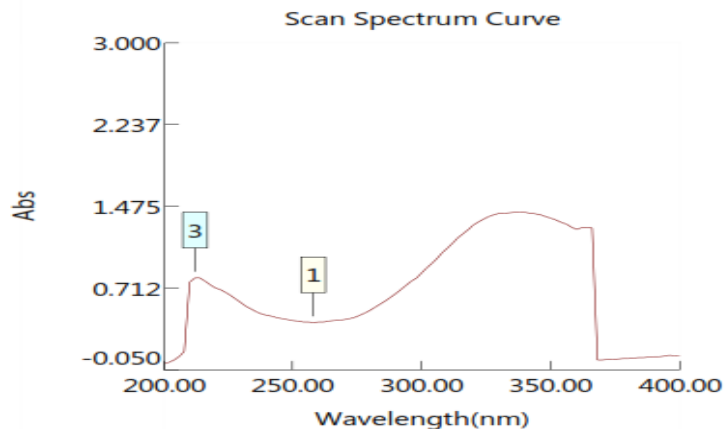
Figure 2: Spectrum showing overlapping spectrum of BEM and EZE
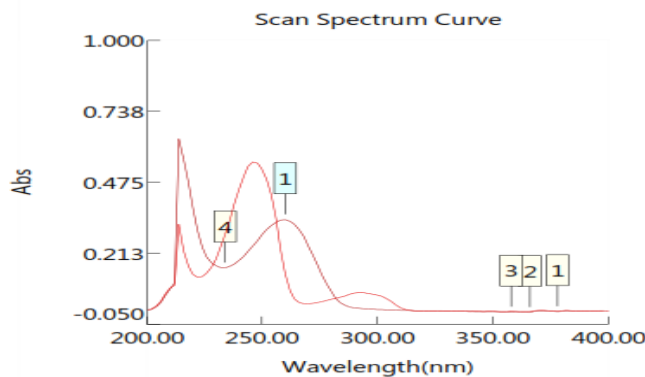
Figure 3: Spectrum showing wavelength of Bempedoic acid

Figure 4: Spectrum showing wavelength of Ezetimibe

The chromatographic method development for the simultaneous estimation of Bempedoic acid and Ezetimibe were optimized by several trials for various parameters as different column, flow rate and mobile phase, finally the following chromatographic method was selected for the separation and quantification of

Bempedoic acid and Ezetimibe in API and pharmaceutical dosage form by RP-HPLC method.

VALIDATION REPORT

Specificity: The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The study was performed by injecting blank. The specificity test was performed for Bempedoic acid and Ezetimibe. It was found that there was no interference of impurities in retention time of analytical peak.
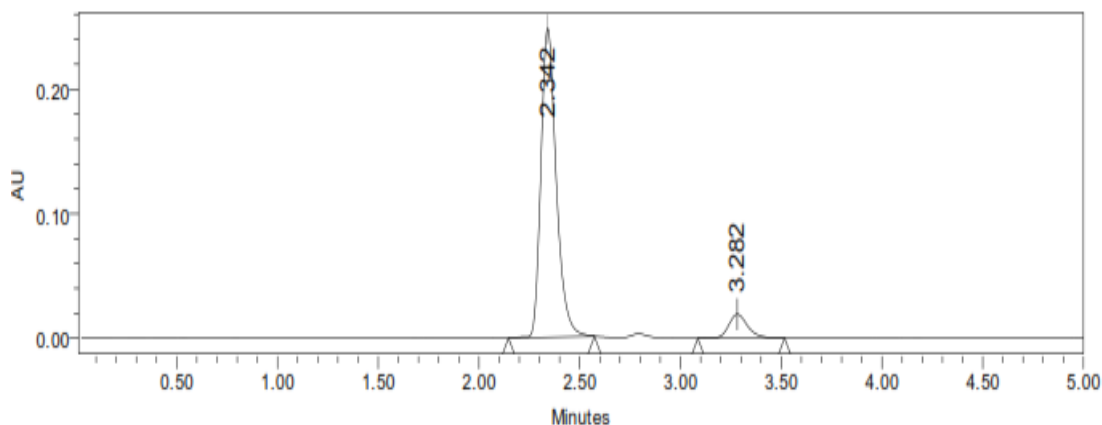
Figure 5: Chromatogram showing blank (mobile phase preparation)
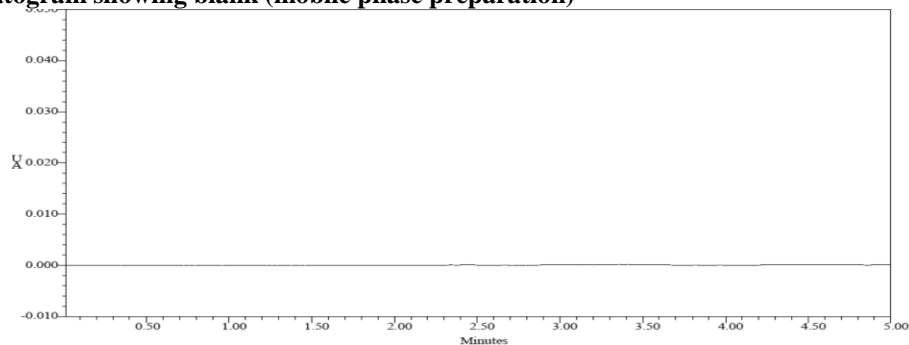
Figure 6: Chromatogram showing standard injection
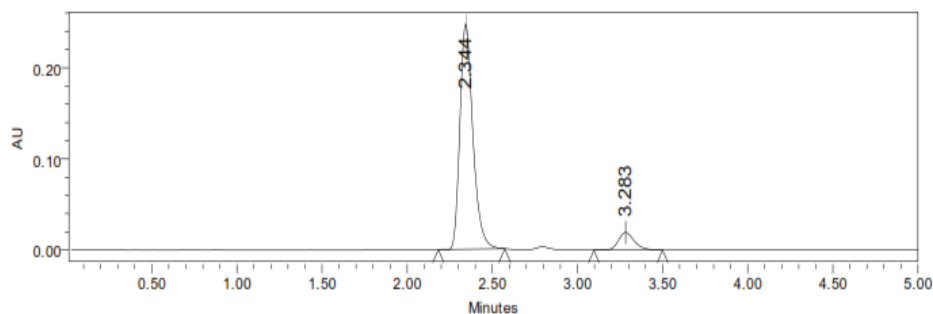
**Figure 7: Chromatogram showing sample injection**

Linearity

The linearity study was performed for the concentration of 50 ppm to 250 ppm and 5ppm to 25 ppm level. Each level was injected into chromatographic system. The area of eac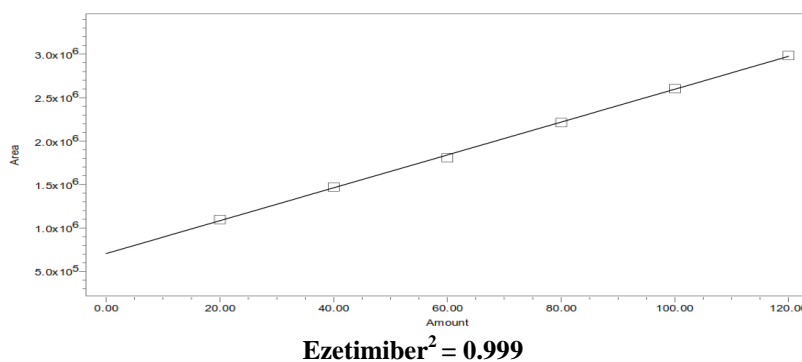h level was used for calculation of correlation coefficient. The linearity study was performed for concentration range of 50.µg-250µg and 5µg-50µg of Bempedoic acid and Ezetimibe and the correlation coefficient was found to be 0.999 and 0.999.(NLT 0.999).

Table 2: Linearity Results for Bempedoic acid

S.No	Linearity Level	Concentration	Area
1	I	50 ppm	471543
2	II	100 ppm	656277
3	III	150 ppm	794999
4	IV	200 ppm	946124
5	V	250 ppm	1002139
Correlation Coefficient			0.999

Figure 8: showing Calibration graph Bempedoic acid

Bempedoic acid $r^2 = 0.999$
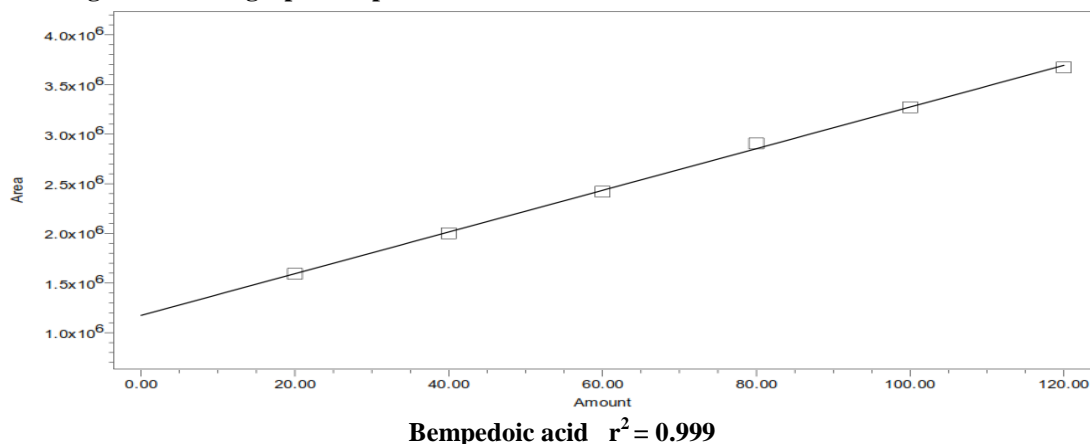
Table 3: Linearity Results for Ezetimibe

S.No	Linearity Level	Concentration	Area
1	I	5ppm	56472
2	II	10 ppm	73841
3	III	15ppm	92655
4	IV	20ppm	111541
5	V	25ppm	130567
Correlation Coefficient			0.999

Figure 9: Showing calibration graph for Ezetimib

Ezetimib$r^2 = 0.999$

Accuracy

The accuracy study was performed for 50%, 100% and 150 % for Bempedoic acid and Ezetimibe. Each level was injected in triplicate into chromatographic system. The area of each level was used for calculation of % recovery.

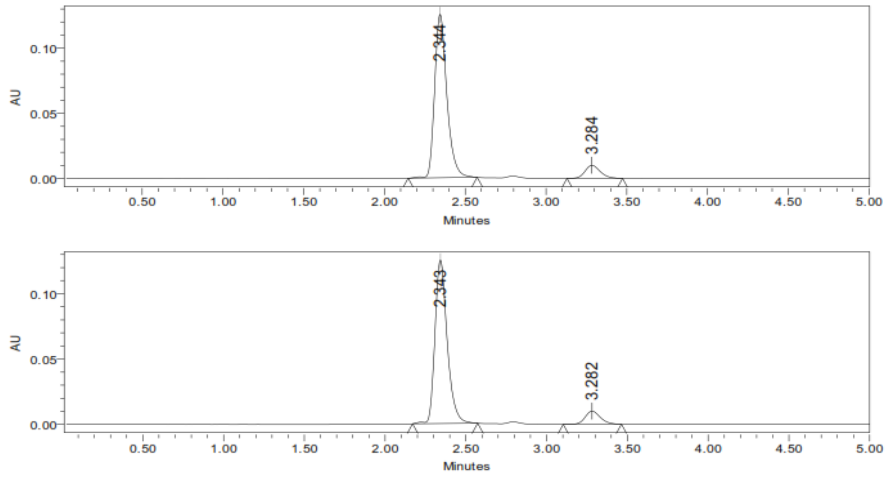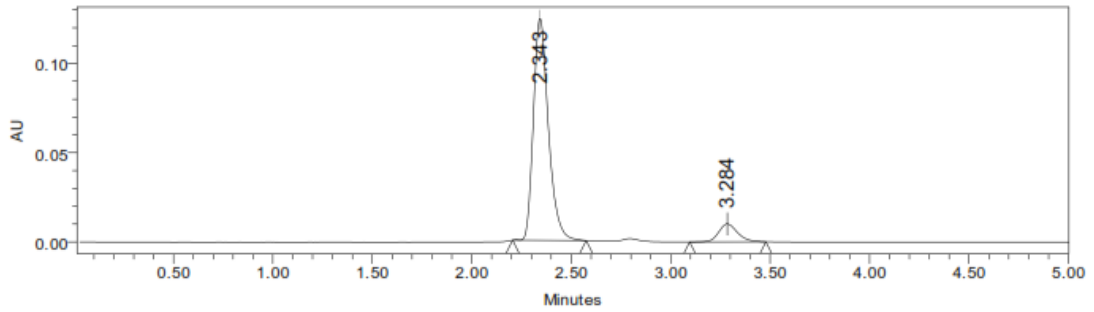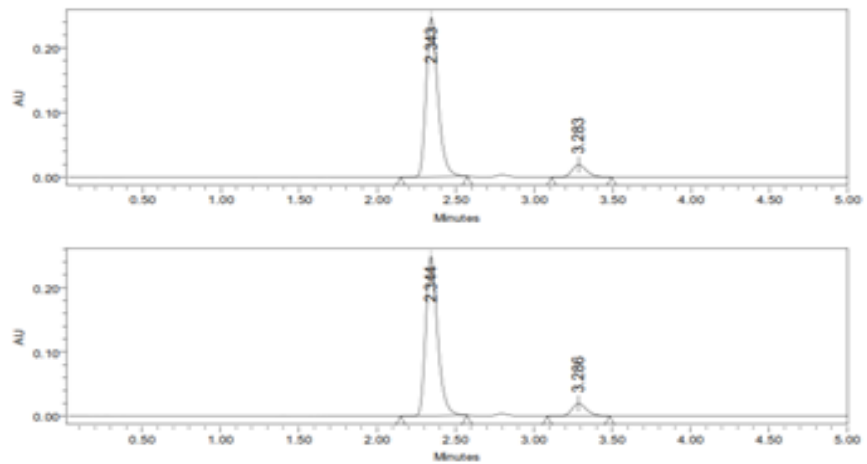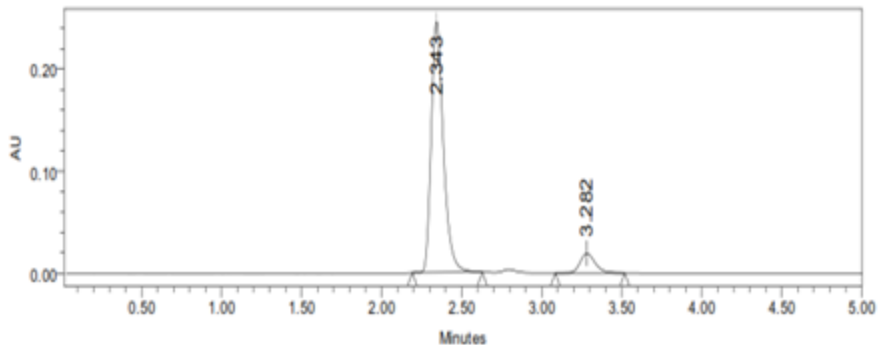**Figure 10: Chromatograms showing accuracy-50% injection-1,2,3**

Figure 11: Chromatogram showing accuracy -100% injection-1,2,3.

Accuracy -100%

Table 4: Showing accuracy results for Bempedoic acid

%Concentration (at specification level)	Average area	Amount added (mg)	Amount found (mg)	% Recovery	Mean recovery
50%	656659	5	4.96	99.91%	99.56%
100%	1304258	10	9.98	99.18%	
150%	1854608	15	15.02	99.60%	

Table 5: Showing accuracy results for Ezetimibe

%Concentration (at specification level)	Average area	Amount added (mg)	Amount found (mg)	% Recovery	Mean recovery
50%	65312	0.5	0.99	99.53%	99.47%
100%	124509	1.0	1.05	99.38%	
150%	178517	1.5	1.495	99.52%	

Precision

Repeatability:The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Table 6: Showing% RSD results for Bempedoic acid

Peak Name: Bempedoic acid

	Peak Name	RT	Area (µV*sec)	Height (µV)
1	Bempedoic	2.343	1302729	248455
2	Bempedoic	2.344	1309759	248699
3	Bempedoic	2.344	1302947	249526
4	Bempedoic	2.345	1303977	246695
5	Bempedoic	2.345	1303236	250012
Mean			1304529.8	
Std. Dev.			2961.1	
% RSD			0.2	

Table 7: Showing %RSD results for Ezetimibe

Peak Name: Ezetimibe

	Peak Name	RT	Area (µV*sec)	Height (µV)
1	Ezetimibe	3.285	124263	19458
2	Ezetimibe	3.287	124487	19634
3	Ezetimibe	3.287	124175	19600
4	Ezetimibe	3.288	124894	19327
5	Ezetimibe	3.288	124495	19540
Mean			124462.7	
Std. Dev.			278.6	
% RSD			0.2	

Intermediate precision/Ruggedness:The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Detection limit

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Quantitation limit

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Table 8: Showing results for Limit of Quantitation

Drug name	Standard deviation(σ)	Slope(s)	LOQ(μg)
Bempedoic acid	381727.80	583265980	5.80
Ezetimibe	5681.30	469828490	0.212

Robustness

The robustness was performed for the flow rate variations from 0.4ml/min to 0.6ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Bempedoic acid and Ezetimibe.

The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The chromatograms are shown in Fig.No.62-66 and results are tabulated in Table.No.25-28.

Figure 12: Chromatogram showing less flow rate 0.8ml/min
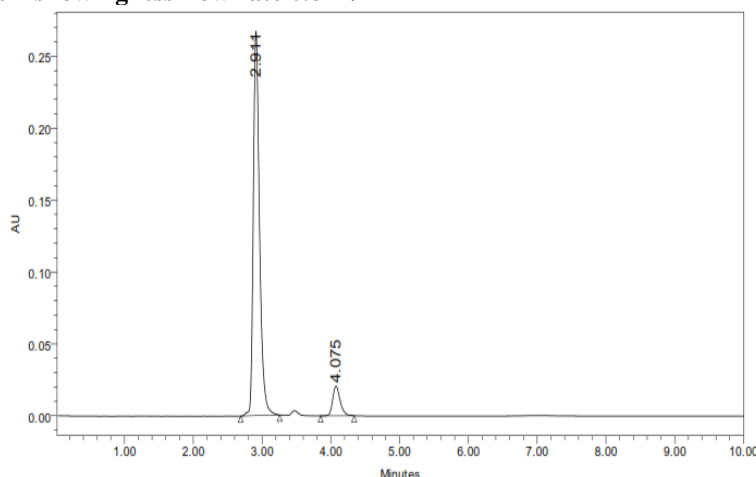
CONCLUSION

A new method was established for simultaneous estimation of Bempedoic acid and Ezetimibe by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Bempedoic acid and Ezetimibe by using ACE C18 column (4.6×150mm) 5μ, flow rate was 1.2 ml/min, mobile phase ratio was (70:30 v/v) methanol:Phosphate buffer pH 3 (pH was adjusted with orthophosphoricacid), detection wavelength was 240nm. The instrument used was Shimadzu, model No. SPD-20MA LC+20AD, Software- LC-20 Solution. The retention times were found to be 2.733 mins and 3.415mins. The % purity of Bempedoic acid and Ezetimibe was found to be 101.27% and 99.97% respectively. The system suitability parameters for Bempedoic acid and Ezetimibe such as theoretical plates and tailing factor were found to be 4668, 1.3 and 6089 and 1. 2, the resolution was found to be 6.0. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study Bempedoic acid and Ezetimibe was found in concentration range of 50μg-250μg and 5μg-50μg and correlation coefficient (r2) was found to be 0.999 and 0.999, % recovery was found to be 99.56% and 99.48%, %RSD for repeatability was 0.2 and 0.2, % RSD for intermediate precision was 0.2 and 0.1respectively. The precision study was precise, robust, and repeatable.LOD value was 3.17 and 5.68, and LOQ value was 0.0172 and 0.2125 respectively.

Hence the suggested RP-HPLC method can be used for routine analysis of Bempedoic acid and Ezetimibe in API and Pharmaceutical dosage form

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