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Research Article

Stability Indicating Method Development and Validation for the Estimation of Bempedoic Acid and Ezetimibe by Reverse Phase–Ultra Performance Liquid Chromatography

Divya Molleti, Krishnamanjari P. Amgoth*, Sushma Pallekona

A.U. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India

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ABSTRACT

A simple, accurate, precise method was developed to simultaneously estimate the bempedoic acid (BEM) and ezetimibe (EZT) in bulk and pharmaceutical dosage form using RP-UPLC. Combination of bempedoic acid and ezetimibe is used to treat adults with heterozygous familial hypercholesterolemia or established atherosclerotic cardiovascular disease. Chromatographic separation was carried out using X-Bridge phenyl 150 x 4.6 mm, 3.5 µ column at a 1.0 mL/min flow rate. The mobile phase consisted of 0.1% formic acid buffer and acetonitrile (70:30%v/v). The retention times of BEM and EZT were found to be 1.157 and 3.208 minutes, respectively. The temperature was maintained at ambient. The optimized wavelength for BEM and EZT was 230 nm. The method developed has been statistically validated according to ICH guidelines. %RSD of BEM and EZT were and found to be 0.6 and 1.4, respectively. %Recovery was obtained as 100.0% and 101.40% for BEM and EZT, respectively. LoD, LoQ values were obtained from regression equations of Bempedoic acid and Eetimibe were 0.27 µg/mL, 0.015 µg/mL and 2.7 µg/mL, 0.15 µg/mL respectively. The method obeys Beer's law in the concentration range of 27–337.5 µg/mL (R2 = 0.9991) for BEM, and 1.5–18.75 µg/mL (R2 = 0.9993) for EZT. The method showed good reproducibility and recovery with %RSD less than 2. Forced degradation studies established the stability-indicating capability of the method under stress conditions like acid, base, peroxide, UV, thermal, humidity. Retention times were decreased and that run time was decreased. Hence, the chromatographic method developed was simple and economical and can be adopted in regular quality control test in Industries. It is said to be rapid, specific, sensitive, robust, and reliable and can be effectively applied for routine analysis in research institutions, quality control departments in industries.

Introduction

A combination of bempedoic acid and ezetimibe is used to treat adults with heterozygous familial hypercholesterolemia or established atherosclerotic cardiovascular disease.[1] Bempedoic acid and ezetimibe were approved on 26 February, 2020 for increased control of LDL cholesterol levels in patients experiencing refractory elevations despite previous statin treatment.[2]

Bempedoic acid is sold under brand name of Nexletol. It is indicated in tolerated statin therapy to treat adults with heterozygous familial hypercholesterolemia or established atherosclerotic cardiovascular disease requiring additional LDL-C lowering.[3] Bempedoic acid is a first-in-class adenosine triphosphate-citrate lyase (ACL) inhibitor used once a day to reduce LDL cholesterol levels in statin-refractory patients. It was developed by Esperion Therapeutics Inc. and approved by the FDA on 21 February, 2020.[4] Ezetimibe is an oral medication used to treat high blood cholesterol and other lipid abnormalities.[5] Ezetimibe is used as adjunctive therapy to a healthy diet for lowering the cholesterol levels in primary hyperlipidemia, mixed hyperlipidemia, homozygous familial hypercholesterolemia (HoFH), and homozygous sitosterolemia (phytosterolemia).[6] The chemical structures of BEM and EZT are given in

***Corresponding Author:** Dr. Krishnamanjari P. Amgoth

Address: A.U. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India

Email ✉**:** akmpawar@andhrauniversity.edu.in

Tel. : +91-8099125548

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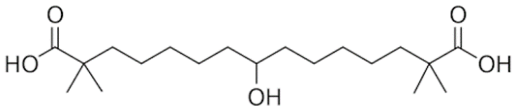

Fig. 1: Structure of Bempedoic acid

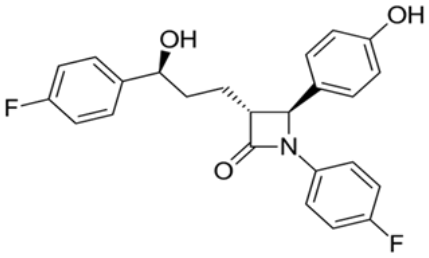

Fig. 2: Structure of Ezetimibe

Fig. 1 and 2 respectively. Extensive literature survey was conducted to find out the analytical methods reported for bempedoic acid and ezetimibe. A literature survey discloses that no UPLC method for estimating bempedoic acid and ezetimibe combined dosage form has been reported until now. Few stability-indicating UPLC, RP-HPLC methods have been reported for the estimation of ezetimibe along with other drug combinations in pharmaceutical preparations.[7-11] this study aims to develop and validate a stability-indicating method with less runtime, which would be able to separate and quantify a combination of BEM and EZT in a single run. The developed method was validated as per ICH guidelines[12,13] and can be applied successfully to quality control determinations.

Materials and Methods

Chemicals and Reagents

Bempedoic acid and ezetimibe reference standards were procured from Glenmark pharmaceutical private limited, Andheri (E), Mumbai, India. HPLC grade acetonitrile, analytical grade formic acid**,** hydrochloric acid, sodium hydroxide, and hydrogen peroxide were from Ramkem (Haryana, India). Milli-Q-water was used throughout the process.

UPLC Method Development

Instrumentation

Waters UPLC 2695 System equipped with quaternary pumps, photodiode array detector, and auto sampler integrated with Empower 2 Software was used in the current investigation. Ultrasonic bath (Labman Scientific Instruments Pvt. Ltd. Chennai, India) for sonication and digital pH meter (Metsar Technologies Pvt. Ltd. Hyderabad, India) for measuring pH were used in the study. Electronic balance ELB 300 was used for weighing the materials. For degradation studies, hot air oven and a UV cross inker, with series of 23400 model UV chambers, equipped with a UV fluorescence lamp with a wavelength range between 200 & 300 nm were used.

Chromatographic Conditions

UPLC analysis was carried out on Waters UPLC System equipped with the 2695-separation module connected to 2996-photo diode array detector and the data was acquired by Empower® version 2. Separation was achieved using X-Bridge phenyl 150 × 4.6 mm, 3.5 µ analytical column with a mobile phase of 0.1% formic acid buffer and acetonitrile in the ratio of 70:30. The samples were analyzed using 10 µL injection volume, flow rate was maintained at 1.0 mL/min with a runtime of 5 minutes, and the ambient temperature was maintained throughout the analysis. Detection and purity establishment of the drugs was achieved using PDA detector at 230 nm wavelength.

Preparation of Working Standard Solutions

Accurately weighed and transferred 270 mg of bempedoic acid and 15 mg of ezetimibe working standards into a 100 mL clean dry volumetric flask, added 50 mL of diluent, and sonicated for 30 minutes to dissolve completely. The final volume was made with the diluent to obtain a concentration of 2700 µg/mL of bempedoic acid and 150 µg/mL of ezetimibe. 5 mL was pipetted out into a 50 mL volumetric flask from the above stock solution and then made up to the final volume with diluent. The final concentration was found to be 270 µg/mL of bempedoic acid and 15 µg/mL of ezetimibe.

Preparation of Sample Solution

Ten tablets were weighed and calculated the average weight. Tablets were crushed in mortar and pestle and the weight equivalent to one tablet (396 mg) was transferred into a 100 mL volumetric flask. To it 70 mL of diluent was added and sonicated for 30 minutes. The further volume was made up with diluent and then filtered with 0.45 µ syringe filter. From the above stock solution, 5 mL was pipetted out and taken into a 50 mL volumetric flask and made up with diluent to obtain a final concentration of 270 µg/mL of bempedoic acid 15 µg/mL of ezetimibe.

Method Validation

The developed and optimized RP-UPLC method was validated according to international conference on harmonization (ICH) guidelines Q2 (R1) in order to determine the system suitability, linearity, precision, the limit of detection (LoD), the limit of quantification (LoQ), accuracy, ruggedness, and robustness.

System Suitability

System suitability parameters were evaluated to verify the system performance. 10 µL of the standard solution was injected five times into the system, and the chromatograms were recorded. Parameters such as the number of theoretical plates and peak tailing were determined and all the parameters obtained were within limits.

Specificity

The specificity of the analytical method was established by injecting the solutions of diluent (blank), placebo, working standards and sample solution individually to investigate the interferences from the representative peaks.

Precision

Method precision/repeatability was performed by injecting six BEM, and EZT replicates into the system and calculating the %assay and %RSD for each compound. Reproducibility/Ruggedness/Intermediate precision was performed using different analysts and a different instrument in the same laboratory.

Accuracy

The accuracy of the proposed method was determined using recovery studies by the spiking method. The recovery studies were carried out by adding known amounts (50, 100, and 150%) of the working standard solution to the pre-analyzed sample. The solutions were prepared in triplicates to determine the accuracy.

Linearity

Linearity was evaluated by analyzing different concentrations of the standard solutions of BEM and EZT. Six working standard solutions were prepared and injected, ranging between 27–337.5 µg/mL for BEM and 1.5–18.75 µg/mL for EZT. The response was a linear function of concentration over mean peak area and was subjected to linear least-squares regression analysis to calculate the calibration equation and correlation coefficient.

Limit of Detection and Limit Of Quantification

The calibration curve method determined the limit of detection (LoD) and limit of quantification (LoQ) of BEM and EZT. Solutions of BEM and EZT were prepared in linearity range and injected (n = 3). Average peak areas were plotted against concentration.

Robustness

To examine the robustness of the developed method, experimental conditions were deliberately changed, and the resolution, tailing factor, and theoretical plates of BEM and EZT peaks were evaluated. To study the outcome of the flow rate on the developed method, it was changed ± 0.1 mL/min and the mobile phase composition was changed ±10 % from the initial composition of the organic phase. In all the above varied conditions, the aqueous component of the mobile phase was held constant.

Forced Degradation Studies

Stress studies were performed by considering the standard working solutions of concentrations 270 µg/mL of BEM and 25 µg/mL of EZT to provide the stability-indicating property of the proposed method. Intended degradation was attempted by the stress conditions of exposure to photolytic stress (1.2 million lux hours followed by 200 Watt hours), heat (exposed at 105°C for 6 hours), acid (1N HCl for 30 min at 60°C), base (1 N NaOH for 30 minutes at 60°C), oxidation (30% peroxide for 30 minutes at 60°C), and water (refluxed for 12 hours at 60°C). The solutions were injected into the system; chromatograms were recorded to assess the stability of sample.

Results and Discussion

Validation Parameters

System Suitability

The column efficiency for BEM and EZT peaks was identified from the theoretical plate count of more than 3000 and the tailing factor was less than 2.0. %RSD for peak areas from six replicate injections was found to be less than 2.0 %. The results of other system suitability parameters, such as, resolution, peak tailing, and theoretical plates, are presented in Table 1. All system-suitable parameters were found to be satisfactory.

Specificity

From the obtained chromatograms in Figs 3 to 6, there were no co-eluting peaks at the retention time of BEM and EZT, which shows that the peak of the analyte was pure and the excipients in the formulation did not interfere with

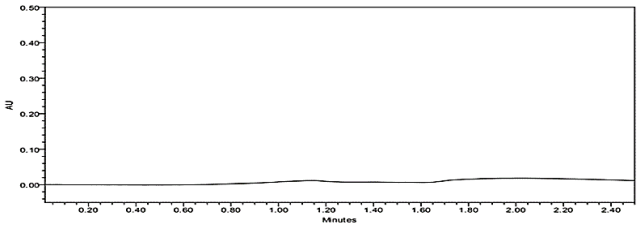

Fig. 3: Chromatogram of blank

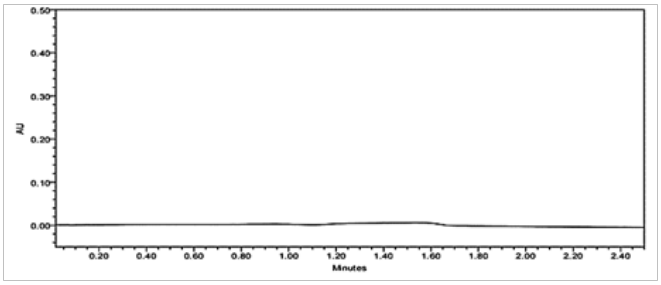

Fig. 4: Chromatogram of placebo

Table 1: System suitability data

Parameter	*BEM*	*EZT*	*Acceptance criteria*
USP Plate count*	2539	4444	NLT 3000
%RSD	0.6	1.4	NMT 2.0
Peak Tailing*	1.05	1.0	NMT 2.0
Resolution*	--	7.78	NLT 1.5

* = Average of 6 replicate injections

the analyte of interest. Figs 3 and 4 show no interference of blank and placebo at the retention time of BEM and EZT from the other excipients.

Method Precision

%Assay for BEM and EZT were in the range of 98–102%. The %RSD for BEM and EZT were found to be within 2%. Hence the method is precise, reproducible, and rugged for 48 hours study and the results are summarized in Table 2.

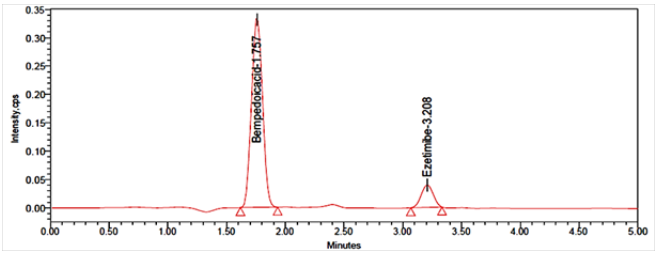

Fig. 5: Chromatogram of Bempedoic acid and Ezetimibe standards

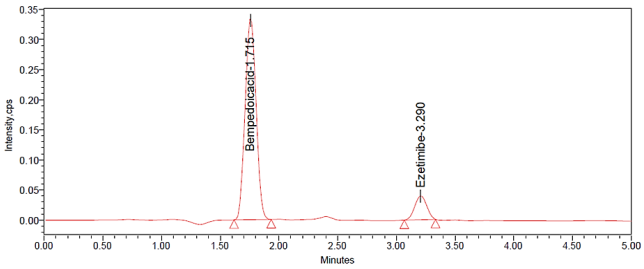

Fig. 6: Chromatogram of Bempedoic acid and Ezetimibe sample

Table 2: Method precision data

	Peak Areas	*%Assay*	*Peak Areas*	*%Assay*
S. No.	*BEM*		*EZT*	
1	2159715	99.9	271128	98.5
2	2180789	100.8	272087	98.8
3	2169223	100.3	274278	99.6
4	2145136	99.2	275687	100.1
5	2169054	100.3	271713	98.7
6	2151732	99.5	272174	98.9
Mean	2162608	100	272844	99.1
SD	13021.4	0.587	1754.1	0.616
% RSD	0.60	0.59	0.64	0.62

Table 3: Accuracy data

Drug name	*Conc. (%)*	*Amount spiked (µg/mL)*	*Amount recovered (µg/mL)*	*%recovery*	*Statistical parameters*
BEM	50	135	135.74	100.6	Mean%: 100.0
	100	270	266.06	98.5	SD: 1.343
	150	405	409.23	101.0	%RSD: 1.34
EZT	50	7.5	7.57	101.0	Mean%: 101.4
	100	15	15.20	101.4	SD: 0.351
	150	22.5	22.87	101.7	%RSD: 0.35

Accuracy

The %recovery for BEM and EZT was within the range of 98–102%. The %RSD for BEM and EZT was found to be within 2%. Hence the proposed method was accurate, and the results are summarized in Table 3.

Linearity

Linearity was evaluated by analyzing different concentrations. The correlation coefficient obtained was greater than 0.999 for all the components. The slope and y-intercept values were also provided in Table 4, which confirmed good linearity between peak areas and concentration. The linearity graphs of BEM and EZT are shown in Figs 7a and 7b, respectively.

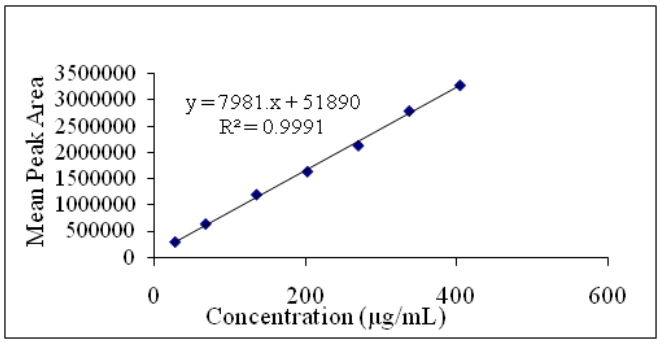

Fig. 7a: Standard curve of Bempedoic acid

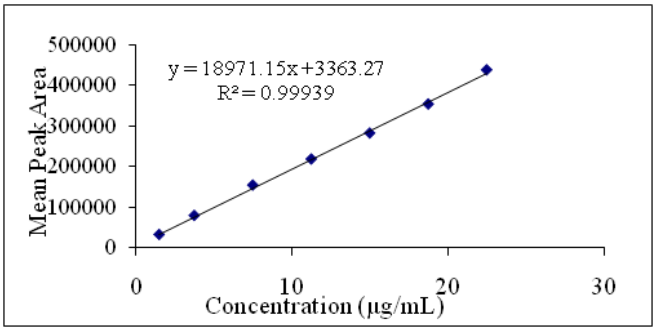

Fig. 7b: Standard curve of ezetimibe

Table 4: Linearity data

	BEM		EZT	
S. No.	Concentration (µg/mL)	Peak area*	Concentration (µg/mL)	Peak area*
1	27.00	28317	1.50	30812
2	67.50	628487	3.75	77837
3	35.00	119250	7.50	152790
4	202.50	1631664	11.25	216985
5	270.00	2130781	10.00	281081
6	337.50	2793848	18.75	352775
	Regression equation $y = 7981x + 51890$ $R^2 = 0.9991$		Regression equation $y = 18971.15x + 3363.27$ $R^2 = 0.9993$	

* = Average peak area of 6 replicate injections for each concentration

Table 5: LoD and LoQ data

Drug name	*LoD (µg/mL)*	*LoQ (µg/mL)*
BEM	0.27	2.7
EZT	0.015	0.15

Table 6: Robustness data

Parameter		*System Suitability Parameters*				
		RT (min)	*Plate count*	*Peak tailing*	*Resolution*	*%RSD*
Optimized method	BEM	1.757	2533	1.05	-	0.60
	EZT	3.208	4562	1.06	7.75	1.40
Flow rate (0.9 mL/min)	BEM	2.165	2194	1.07	-	0.07
	EZT	3.963	5668	1.04	8.98	0.31
Flow rate (1.1 mL/min)	BEM	1.470	2102	1.02	-	0.51
	EZT	2.672	3190	1.04	6.57	0.31
Decrease in organic phase (65:35)	BEM	1.858	2648	1.07	-	0.07
	EZT	4.057	5604	0.96	10.92	0.45
Increase in organic phase (75:25)	BEM	1.681	2479	1.03	-	0.59
	EZT	2.761	3681	0.98	5.60	0.98

Table 7: Forced degradation studies at different stress conditions

Type of degradation	*Bempedoic acid*			*Ezetimibe*		
	Peak area	*Recovered (%)*	*Degraded (%)*	*Peak area*	*Recovered (%)*	*Degraded (%)*
Acid	2068594	95.60	4.40	265864	96.40	3.60
Base	2069532	95.60	4.40	267864	97.00	3.00
Neutral	2162439	99.90	0.10	275486	99.80	0.20
Peroxide	2086592	96.40	3.60	265342	96.20	3.80
Thermal	2074512	95.80	4.20	266542	96.60	3.40
UV light	2082647	96.20	3.80	266821	96.70	3.30
Reduction	2082563	96.20	3.80	268582	97.30	2.70

LoD and LoQ

The Limit of Detection and Limit of Quantification of BEM and EZT were calculated by using the following equations (ICH, Q2 (R1)). The LoD and LoQ values are reported in Table 5.

$$\text{These LoD} = 3.3 \times \sigma/S \text{ and LoQ} = 10 \times \sigma/S$$

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

Robustness

The system suitability parameters such as resolution, RSD, tailing factor, or the theoretical plates of BEM and EZT remained unaffected by deliberate changes. The results were presented in Table 6, along with the system suitability parameters of normal conditions. Thus, the method was found to be robust concerning variability in applied conditions.

Forced Degradation Studies

Blank, placebo, and degradation samples were analyzed with the above-mentioned UPLC conditions using a PDA detector to monitor the homogeneity and purity of the BEM and EZT. Degradation was not observed in photolytic stress, humidity, acid, base, water hydrolysis, and thermal stress studies. It was interesting to note that all the peaks due to degradation were well resolved from the peaks of BEM and EZT. Further, the peak purity of BEM and EZT was homogeneous based on the evaluation parameters such as purity angle and purity threshold. The verification of peak purity indicates no interference from degradants, facilitating error-free quantification of BEM and EZT. Hence, the method is considered to be "stability-indicating." The obtained results are shown in Table 7.

Conclusion

A simple and rugged RP-UPLC method has been developed to simultaneously determine bempedoic acid and ezetimibe in active pharmaceutical ingredients and tablet formulation Nexlizet. The proposed method was validated according to ICH guidelines by testing its parameters, including system suitability, specificity, linearity, LoD, LoQ, precision, accuracy, and robustness.

The method was specific to separate the peaks of active pharmaceutical ingredients from the degradation products obtained with good resolution after forced degradation studies.

Thus, stress made studies evidence the effectiveness of the proposed stability-indicating RP-UPLC method, which can be adopted in routine analysis in pharmaceutical industries.

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