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RP-HPLC METHOD VALIDATED BY QUALITY BY DESIGN FOR ESTIMATION OF BEMPEDOIC ACID IN BULK AND PHARMACEUTICAL FORMULATION

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ABSTARCT

The research presents a method for developing and validating analytical methods for treating newer high-level low-density lipoproteins (LDL) using Bempedoic acid, which is an alternative treatment for primary hyperlipidaemia in patients. The objective of this study was to provide an easy, fast, sensitive, accurate, precise, and economical technique for estimating Bempedoic acid in pharmaceutical and bulk items by The proposed method made use of a Acetonitrile: Water 70:30% v/v mobile phase with an optimum flow rate of 1.0 ml/min via Box Behnken Design Expert 7.0. Inertsil ODS-3V (150 mm X 4.6 mm i.d.) 5 µm C18 column used for method development and wavelength detection at 212 nm. The retention time of Bempedoic acid was 4.25 minutes. After developing a method according to ICH guidelines, validation is complete. The linearity of the correlation coefficient of the developed method was found to be 0.996. The limit of detection (LOD) and limit of quantification (LOQ) for Bempedoic acid were found to be 16.34 and 49.52 µg/ml respectively. For RP-HPLC, several system suitability parameters were found to be specific according to ICH guidelines, viz. tailing, theoretical plates, resolution. The QBD approach was with RP-HPLC during method development and helps make all methods sensitive and cost-effective.

Keywords: Bempedoic Acid, RP-HPLC, Quality by Design, Box Behnken Design, Development, Validation

INTRODUCTION

A recently discovered oral inhibitor of adenosine triphosphate (ATP) citrate lyase (ACL) is Bempedoic Acid **[1]**. The Food and Drug Administration (FDA) has approved the drug for use in 2020 in the case of adults who need additional lowering of their low-density lipoprotein cholesterol **[2]**. As a prodrug, Bempedoic acid needs to be activated in the liver. The enzyme very-long-chain acyl-CoA synthetase-1 (ACSVL1) is responsible for activating structurally Bempedoic acid, also known as 8-hydroxy-2,2,14,14 tetramethylpentadecanedioic acid, to ETC-1002-CoA, the pharmacologically active metabolite. An essential function of ATP lyase is in the production of cholesterol. Once coenzyme A (CoA) has activated the parent drug in the liver, ETC-1002-CoA directly inhibits this enzyme **[3]**. The chemical structure of Bempedoic acid shown in **Figure 1**.

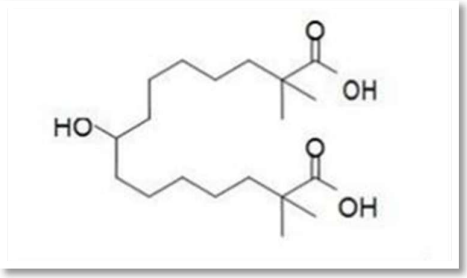


Figure 1: Bempedoic Acid

A broad literature survey for Bempedoic acid that several methods based on varied techniques are available for individual and combination drugs. Some recently reported methods which are HPLC **[10]**, RP-HPLC **[11-17]**, LC-MS **[18]**, RP-UPLC **[19]**, Stability Indicating **[20-21]**, was traced out by effective literature survey.

According to the information from literature survey, there is not even a single method reported for the reverse phase high performance liquid chromatography (RP-HPLC) of Bempedoic acid using the Quality by Design in the pharmaceutical formulation. So, research was focused to develop a new RP-HPLC method using QbD with good sensitivity, simple and economical mobile phase, accuracy and reliable precision also cost-effective method for routine analysis.

MATERIALS AND METHODS

1. Chemical & Reagent

Bempedoic acid was procured from Vidisha Analytical Laboratory in India. The tablet purchased from local markets was labeled with 180 mg of Bempedoic acid. HPLC grade chemicals and reagents used for Methanol Acetonitrile were collected from Merck lab. Siddhi lab provided HPLC-grade water throughout the procedure.

2. Equipment

The RP-HPLC analysis was carried out employing the Agilent 1260 Infinity II HPLC on a reverse-phase Inertsil ODS-3V C 18 column having dimensions of 150 mm x 4.6 mm i.d. and a particle size of 5 µm. The DEACX16446 detector was used.

Spectrophotometric measurements were carried out using a double-beam UV visible spectrophotometer (Jasco UV 550). The analytical Azcet high precious balance, model CY 224C, was used. For Sonicator purposes, a Bio-Technic Ultra Sonicator is used.

QbD Software- All the computations for the current optimization study and statistical analysis were performed using Design Expert® software (Design Expert version 7.0.0; State-Ease Inc., Minneapolis, MN, USA).

3. Preparations of Solutions

3.1. Preparation of Standard Stock Solution:

Bempedoic acid Standard stock solution was prepared by dissolving 20 mg Bempedoic acid into a 10 ml clean and dried volumetric flask, added about 7 ml of methanol to dissolve it completely and made volume up to the mark with methanol (2000 PPM). Further diluted 5 ml to 10 ml with water (1000 PPM).

3.2. Determination of Detection Wavelength:

Between 200 and 400 nm, the standard solution was scanned, the examination was selected from the wavelength of maximum absorption from the spectrophotometric analysis and it was 212 nm**.** shown in **Figure 2**.

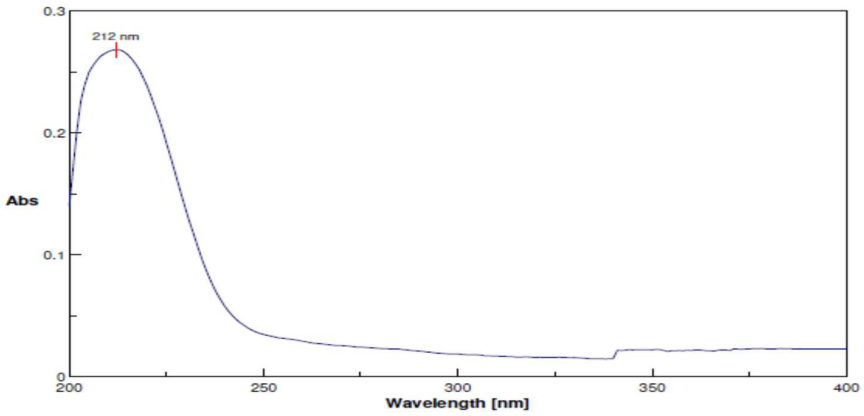


Figure 2: UV Spectrum of Bempedoic Acid

3.3. Chromatographic Condition

The mobile phase was consisting of Acetonitrile: Water (70:30)% v/v, and it was filtered and sonicated prior to used. For the stationary phase Inertsil ODS-3V C18 column having dimensions of 150 mm x 4.6 mm i.d. was used. The column oven temperature was kept at 35^0C and the flow rate was maintained at 1.0ml/min. (DEACX16446) detector was used and the UV detection was carried out at 212 nm wavelength and the injection volume was 20 µl.

3.4. Experimental Design

The experimental design was constructed using design expert software version 7.0.0 for the study of different variables and to

verify method performances. The level of these variables is as given in **Table 1**.

3^3 randomized response surface designs with a Box-Behnken design were used with 15 trial runs to study the impact of three factors on the three key response variables. In this design 3 factors were evaluated, each at 3 levels and experimental trials were performed at all 3 possible combinations. The mobile phase composition (X1), flow rate (X2) and column oven temperature (X3) were selected as independent variables and retention time (RT) and asymmetry were selected as dependent variables. The resulting data were fitted into Design Expert 7.0.0. software and analysed statistically using analysis of variance (ANOVA). The data were also subjected to response surface methodology to determine the influence of mobile phase composition, flow rate and column oven temperature on dependent variables.

Table 1: Level of Independent Variables

Level of Variable	Range of Factors		
	Acetonitrile (%v/v)	Flow Rate (ml/min)	Column oven temperature (°C)
Low Level (-1)	65	0.8	32
Medium Level (0)	70	1.0	35
High Level (1)	75	1.2	38

4. Assay of Marketed Test Sample

Weighed 20 tablets transferred in mortar pestle and crushed to fine powder. Mixed the contents with butter paper uniformly. Weighed the powder material equivalent to 100 mg of Bempedoic acid and transferred to clean and dried 50 ml of volumetric flask. Added 35 ml of metahnol, sonicated for 15 minutes with intermittent shaking. After 15 minutes allowed the solution to cool at room temperature and made volume up to the mark with Methanol. Filtered the solution through suitable 0.45 µ syringe filter discarding 3-5 ml of initial filtrate. Further diluted 5.0 ml of filtered stock solution to 10 ml with water. (1000 mcg of Bempedoic acid), injected the resultant solution for RP-HPLC analysis.

5. Method Validation

The method was validated according to ICH Q2(R1) guidelines. Method validation is an analytical process by which it is established by laboratory studios that the performance characteristics of the procedure meet the requirement for the intended analysis application.

5.1. Linearity

Linearity were performed from 10% to 150% of working concentration. Calibration curve was plotted graphically as a function of analyte concentration in µg/ml on X-axis Vs mean area on Y-axis. Resulted values for the correlation coefficient and regression line equation were calculated.

5.2 Accuracy

Accuracy of the method were confirmed by a recovery study from marketed formulation at 3-level of standards range 50%, 100%, 150%. According to ICH recommendations, 98- 102% of standard addition was acceptable for percent recovery.

5.3 Precision

Repeatability, intra-day, and inter-day measurements were used to evaluate the precision of the procedure. The precision measures how closely measurements taken from several samplings of the same homogenous sample under the given circumstances resemble one another. Using three separate analyses of standard drug solutions within each medication's calibration range on the same day, the intra-day precision was ascertained. Analyzing drug solutions throughout the duration of a week on three separate days within the calibration range allowed for the determination of inter-day precision.

5.4 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The lowest drug concentration that can be precisely detected, distinguished from the background is the LOD and quantified at that concentration is known as the LOQ. The following equations were used to calculate LOD and LOQ in accordance with ICH recommendations. LOD = 3.3 × σ/S & LOQ = 10 × σ/S Where σ is standard deviation and S is slope of the calibration curve.

5.5 Robustness

The robustness of the method was calculated by taking different trials through a small change in the chromatographic conditions such as wavelength, flow rate and column oven temperature.

5.6 Specificity

Specificity demonstrate the method's precision, the following solutions will be prepared and injected & Checked peak purity for standard and test sample solution, blank & placebo solutions.

5.7 Solution Stability

The solution was stored at normal illuminated laboratory conditions and analysed after 12 hours and 24 hours. The stability of standard and test solutions was evaluated by determining the variance between the test solution results at each stability time point and the original stability times.

5.8 System Suitability Test

Five repeat studies of Bempedoic acid were used to determine the system suitability. For standard solutions, retention time, peak asymmetries were measured.

RESULT AND DISCUSSION

1. Optimized Chromatographic Method

The proposed work planned for the development and validation of the RP-HPLC method for the estimation of Bempedoic acid in bulk and pharmaceutical formulation. Accordingly, the initial solvent was selected for Bempedoic acid methanol

showed good solubility for it. Future the chromatographic condition was analysed the parameters were finalized and showed a sharp peak in mobile phase Acetonitrile: Water (70:30) % v/v with retention time 4.25 minutes as shown in **Figure 3**.

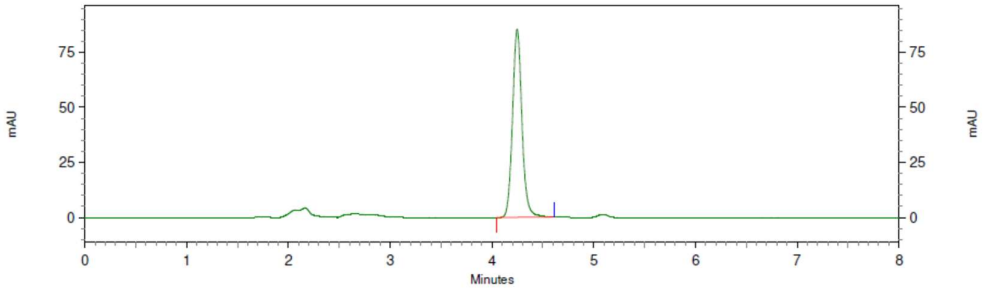


Figure 3: Chromatogram of Bempedoic Acid

2. Method Optimization Using Box Behnken Design (BBD)

The probable 15 trial runs using 3^3 Box Behnken designs. 3center points per block considered for designing the DOE trials under Box Behnken model. Each combination of mobile phase composition % Acetonitrile, Flow rate and COT suggested by BBD were finally run on the system and observed for the responses such as retention time, asymmetry. Run no. 3 selected as an optimized chromatography, as it has Optimum R.T. and asymmetry are shown in following **Table 2**.

Table 2: Box Behnken Design Plan and Responses

Runs	Factor1	Factor 2	Factor3	Response 1	Response 2
	A: % Acetonitrile	B: Flow rate	C: COT (°C)	Retention time (RT)	Asymmetry
1	75	1.0	38	3.74	1.06
2	65	0.8	35	6.21	1.01
3	65	1.2	35	4.12	1.03
4	70	1.0	35	4.25	1.12
5	65	1.0	32	5.00	1.00
6	70	1.2	38	3.49	1.13
7	75	0.8	35	4.74	1.06
8	70	1.0	35	4.25	1.12
9	70	0.8	32	5.39	1.10
10	75	1.0	32	3.81	1.07
11	70	0.8	38	5.28	1.11
12	70	1.0	35	4.25	1.12
13	70	1.2	32	3.57	1.09
14	65	1.0	38	4.92	1.02
15	75	1.2	35	3.13	1.07

3. Statistical Analysis of Method Responses for Retention Time & Asymmetry

The Study employed statistical analysis through the use of analysis of variance (ANOVA) to evaluate the impact of different variables and their interactions on the retention time & asymmetry response. Specific levels in BBD were chosen based on process factors and by determining faced centre values by selecting responses and conducting experiments to which ANOVA is applied of DOE.

Table 3: ANOVA Table for R.T

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	9.83	6	1.64	5296.77	< 0.0001	significant
A-ACETONITRILE	2.92	1	2.92	9425.82	< 0.0001	
B-FR	6.68	1	6.68	21590.34	< 0.0001	
C-COT	0.01	1	0.01	46.71	0.0001	
AB	0.06	1	0.06	186.18	< 0.0001	
A^2	0.05	1	0.051280	165.75	< 0.0001	
B^2	0.12	1	0.123709	399.87	< 0.0001	
Residual	0.00	8	0.000309			
Lack of Fit	0.00	6	0.000413			
Pure Error	0	2	0			
Cor Total	9.8346	14				

Table 4: ANOVA Table for Asymmetry

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.02	2	0.01	85.44	< 0.0001	significant
A-ACETONITRILE	0.01	1	0.01	34.43	< 0.0001	
A^2	0.02	1	0.02	136.45	< 0.0001	
Residual	0.00	12	0.00			
Lack of Fit	0.00	10	0.00			
Pure Error	0.00	2	0.00			
Cor Total	0.03	14				

Table 5: Summary Statistics for Response R1 And R2.

Response	R1	R2
Std. Dev.	0.018	0.012
Mean	4.410	1.074
C.V. %	0.399	1.122
PRESS	0.015	0.0026
R-Squared	0.9997	0.9344
Adj R-Squared	0.9996	0.9234
Pred R-Squared	0.9984	0.9013
Adeq Precision	252.59	18.16

The ANOVA analysis for R1 (R.T) and R2 (Asymmetry) revealed that model terms have significance when the values of 'Prob > F' are below 0.0005. In the case of R1, A, B, C, AB, A2, and B2, significant model terms are shown. In the case of R2, A and A2 are significant model terms. Values greater than 0.1000 indicate that the model terms are not significant. The DOE application resulted in the responses R1 and R2 showing that the models have significant model F-values of 5296.77 and 85.44 respectively. The probability of a "Model F-Value" this large occurring due to noise in both cases is only 0.01%. The Pred R-Squared and Adj R-Squared response values of R1 (0.9984 and 0.9996) and R2 (0.9013 and 0.9234) respectively. Adeq Precision measures the signal-to-noise ratio of R1 252.59 and R2 18.16 shown in **Table no. 3, 4 & 5**. This model can be used to navigate the design space of R.T & asymmetry shown in **Figure 6**.

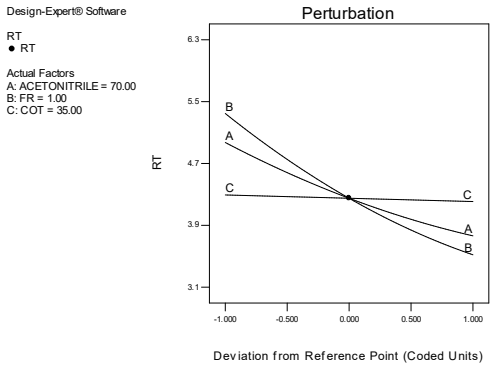


Figure 4: Effect of All 3 factors on R.T

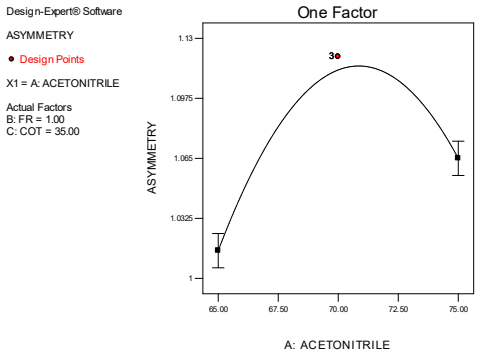
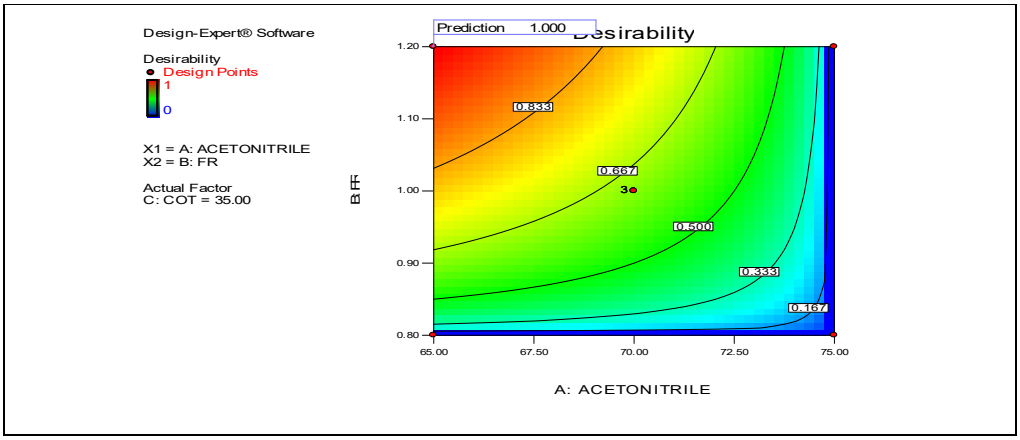


Figure 5: Effect on % Acetonitrile in Mobile phase on Asymmetry

The impact of %Acetonitrile, F.R., and COT in the mobile phase on Retention time is inversely proportional and increases, as shown in **Figure 4**. In regards to the asymmetry effect on %Acetonitrile in the mobile phase, the curvature is directly proportional and has an increasing impact. Flow rate and COT in the mobile phase have no effect on asymmetry, as they were not significant in the ANOVA test as shown in **Figure 5**.

4. Design Space

The optimized chromatographic conditions selected based on the desirability functions approach were mobile phase consisting Acetonitrile:Water (65:35) % v/v of pumped at a flow rate 1.2 ml/min gave the highest desirability of 1. the graph represents the optimized combination of the three selected independent factors, which gave the maximum desirability shown in **Figure 6**.



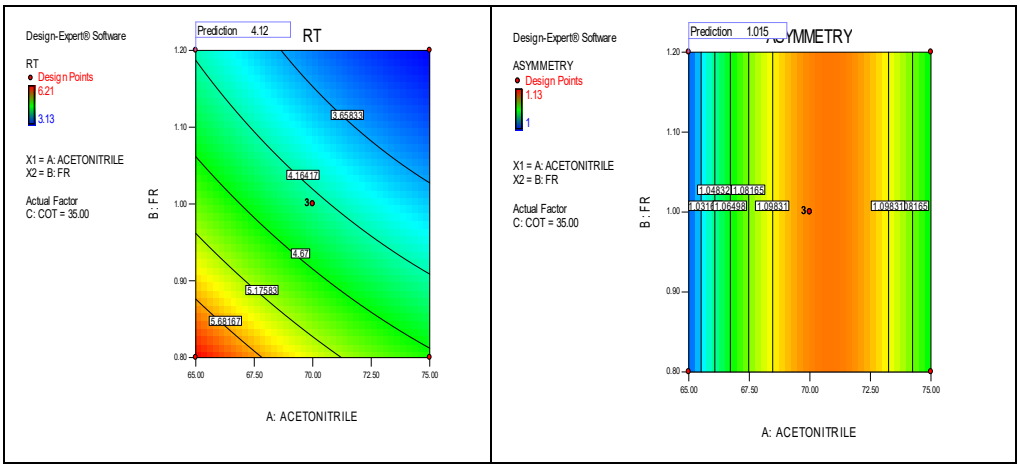


Figure 6: Design Space for Desirability, R.T, Asymmetry

5. Method Validation

5.1 Linearity

The Bempedoic acid showed linearity between concentration range of 100-1500 ppm and had a good correlation coefficient of 0.99996 with slop 7285.90 and y-intercept 43971.22 for the graph of concentration against area result of linearity shown in **Figure 7**.

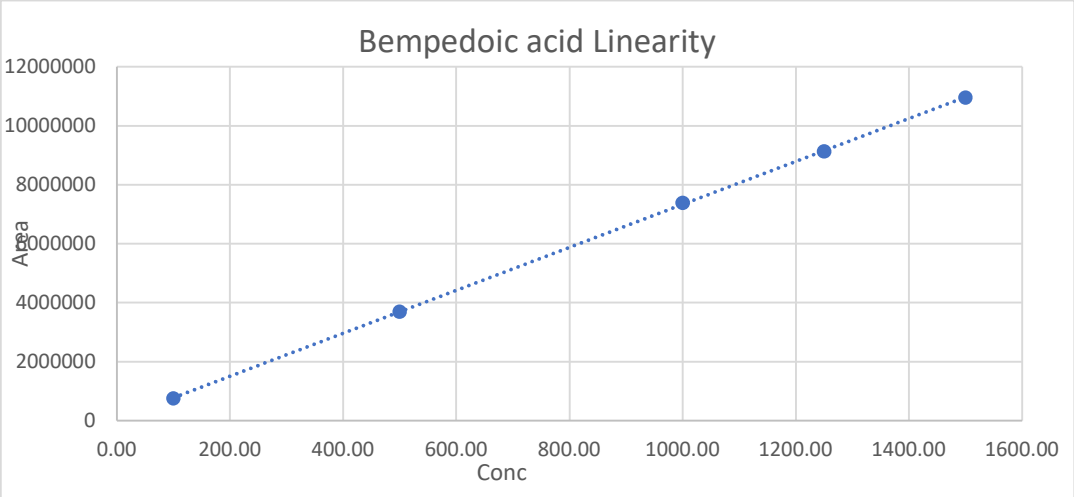


Figure 7: Calibration Curve of Bempedoic Acid

5.2 Accuracy

An analytical method's accuracy is determined by the accuracy of their test results when compared to the true value. The % overall recovery was 99.72 and % RSD was 0.815 which is not more than 2.0%. result of accuracy shown in **Table 6**.

Table.6: Result of Accuracy

Level (%)	Area	Recovered conc (µg/ml)	Added conc (µg/ml)	% Recovery	Mean Recovery	% RSD
50	3680526	498.12	502.00	99.23	99.43	0.892
	3688521	499.21	506.00	98.66		
	3716529	503.00	501.00	100.40		
100	7329524	991.98	1002.00	99.00	99.97	1.024
	7416529	1003.76	1005.00	99.88		
	7451021	1008.42	998.00	101.04		
150	10993245	1487.83	1502.00	99.06	99.77	0.767
	11053651	1496.00	1501.00	99.67		
	11125684	1505.75	1497.00	100.58		

5.3 Precision

The standard deviation or relative standard deviation is commonly used to express the precision of an analytical method. Precision was performed on Test sample. %RSD is not more than 2 result of accuracy shown in **Table 7**.

Table 7: Result of Precision

Sr.no.	Sample	Mean	Standard deviation	%RSD
Repeatability Intra day	Sample 1 to sample 6	97.96	97.96	97.96
Intermediate precision (Inter-Day)	Sample 1 to sample 6	97.92	0.6020	0.6020
Repeatability + Inter-day	Sample 1 to sample 6	97.943	97.943	97.943

5.4 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The Limit of Detection (LOD) was calculated to be 16.34 µg/ml, and the Limit of Quantitation (LOQ) was determined to be 49.52 µg/ml.

5.5 Specificity

Specificity means being able to access the analyte without any doubt, even if there are components that are expected to be present. The peak purity of standard solution was 0.999 & test solution was 0.997.

5.6 Solution Stability

The stability study was carried out under normal laboratory conditions. Standard and Test solution stability study result after 12 hrs was 0.77% & 0.83%. Result after 24 hrs was 1.39% & 1.17%.

5.7 System Suitability Test

Table 8: Result of System Suitability Test

Sr No.	Standard solution	Area	Asymmetry	Theoretical plates
1	Standard_1	7390425	1.03	10956
2	Standard_2	7385428	1.03	10942
3	Standard_3	7390116	1.02	10962
4	Standard_4	7388519	1.03	10967
5	Standard_5	7389423	1.03	10948
Mean		7388782	1.03	10955
STD Dev		2012.981		
% RSD		0.03		

5.8 Robustness

The robustness of an analytical method is based on its ability to remain unaffected by small changes but deliberate changes in method parameters, which indicates its reliability during normal usage shown in **Table 9**.

Table 9: Result of Robustness

Change in Parameter	R.T.	Standard area	Asymmetry	Theoretical plates
Wavelength by +3 nm (215 nm)	4.09	7454986	1.03	10568
Wavelength by -3 nm (209 nm)	4.09	7084320	1.02	10634
Flow rate by +10% (1.32 ml/min)	3.78	6912795	1.02	10330
Flow rate by -10% (1.08 ml/min)	4.46	8191799	1.01	10886
Column oven temp by +2ºC (33 ºC)	4.06	7396521	1.04	10985
Column oven temp by -2ºC (37 ºC)	4.10	7344820	1.05	10719

6. Assay of Marketed Test Sample

The result of marketed test sample assay found to be within acceptable range was 98.26%.

CONCLUSION

Bempedoic acid has been quantified using a cost-effective, sensitive RP-HPLC method using a systematic QbD-based methodology. The method is robust, linear, precise, accurate, and specific, with a lower probability of failure during validation and transfer. The automated QbD method development process using Design Expert software has produced a more robust method in less time. A statistical analysis demonstrates unequivocally that this process is repeatable, simple, robust, and accurate. The pharmaceutical sector will continue to use this technology for routine analysis and quality control since all current technologies have the capacity of performing regular evaluations of Bempedoic acid in tablet form. In the future, research and development (R&D) activities including bioanalytical evaluations associated with RP-HPLC and LC-MS/MS techniques for Bempedoic acid will be largely dependent on QbD.

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