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Development and validation of RP-HPLC method for Azelaic acid in prepared cubosomal dispersion using Design of Expert approach

Kiran chenni[1], Anjana Adhyapak[2]

[1]M. pharm, Department of Pharmaceutical Quality Assurance, KLE College of Pharmacy, Nehru Nagar, KLE Academy of Higher Education and Research, Belagavi, Karnataka, 590010, India
[2]Associate professor, Department of Pharmaceutical Quality Assurance, KLE College of Pharmacy, Nehru Nagar, KLE Academy of Higher Education and Research, Belagavi, Karnataka, 590010, India.
ajanaadhyapak@klepharm.edu

Abstract:

Azelaic acid (AA), a naturally occurring dicarboxylic acid, is used extensively to treat acne and rosacea because of its antibacterial and anti-inflammatory qualities. The objective of this study was to create and validate a straightforward, reliable, and accurate RP-HPLC method for the measurement of AA in bulk and a novel cubosome formulation. Key chromatographic parameters, including the mobile phase composition (50:50 v/v of 0.5% formic acid: acetonitrile), flow rate (1 mL/min), and detection wavelength (210 nm), were chosen to produce the best retention time and resolution. The procedure was optimised using a Box-Behnken Design. The technique showed good accuracy (97–99% recovery), precision (%RSD < 2%), linearity (2–10 µg/mL, R2 = 0.995), and sensitivity (LOD: 1.521 µg/mL; LOQ: 3.342 µg/mL). Studies of forced deterioration under varied stress settings were used to establish stability-indicating capabilities. The verified technique was then used to measure AA in cubosomes, demonstrating favourable particle size (185 nm), zeta potential (-14.36 mV), and good drug entrapment effectiveness (92.4%). This HPLC approach provides a dependable tool for quality control and stability assessment of AA-based medicinal systems and is efficient for routine analysis of AA in pharmaceutical research and formulation development.

Keywords: Azelaic acid, RP-HPLC, Cubosomes, QbD

1. Introduction:

Rosacea is a chronic inflammatory condition that primarily affects the skin regions around the eyes and the centrofacial area, including the cheeks, chin, nose, and forehead.[**1**] People can experience rosacea at any phase of life but the condition typically begins between 30 and 50 years of age. The reported prevalence rates among populations show 1 to 22% variations but the numbers likely reflect differences in research approaches and populations as well as geographical areas and cultural factors influencing the understanding of these conditions.[2,3] Rosacea affects approximately 5.5% of adults globally, according to a systematic research study. This study found no gender-based differences in how rosacea affected men

and women.[4] Previous studies reported women suffered this condition at a greater rate but new findings show men develop it equally often or more frequently.[5]

The identification of rosacea involves three main characteristics: persistent redness, periodic flushing, along with inflammatory papules and pustules and developing telangiectasias. Rhinophyma primarily affects men and typically presents as phymatous alterations, most commonly within the nose. More than half of rosacea patients display eye symptoms such as dryness, foreign body sensations and photosensitivity, and conjunctivitis along with blepharitis with rare instances of keratitis, which threatens eyesight. [6]

Azelaic acid (AA) is a natural dicarboxylic acid that is present in grains and is produced by skin yeast Malassezia furfur. It has anti-inflammatory, antibacterial and keratinization-inhibiting properties and therefore, it is suitable for long-term dermatological use. Azelaic acid (AA) controls acne, rosacea, melasma, and pigmentation of the skin by inhibiting bacterial protein synthesis and the activity of tyrosinase. Its bacteriostatic action brings down both aerobic and anaerobic bacteria including Propionibacterium acnes. Approved by FDA, it inhibits NADPH oxidase and kills reactive oxygen species, thus relieving swelling. [7]

Several analytical methods, including HPLC, HPTLC, HPLC-MS/MS, LC-ESI-MS/MS, UV Spectrometric, and Fluorometric and Colorimetric, have been reported in the literature for the determination of AZA in biological solids (to some extent) and semisolids (to a lesser extent), and in formulations, AZA is more commonly found in fluids than semisolids.[8]

The main aim of the present study was to design a relatively simple, precise, and corroborated visibly standard spectrophotometric techniques for identifying and quantifying Azelaic acid in the purified form and a pharmaceutical dosage form because most previous methods have been discovered to be relatively complex and time-consuming. [9]

2. Material and Methods

Chemicals and chemical agents

Azelaic Acid was procured from OTTO CHEMIE PVT LTD, Mumbai. Hi Media Laboratories Pvt. Limited in Mumbai, India supplied HPLC-grade acetonitrile, methanol, orthophosphoric acid, and formic acid. All chemicals utilised, including Milli-Q Water, poloxomer, and GMO, were obtained from KLE College of Pharmacy in Belagavi, India.

Instrumentational and Analytical Setup

The technique development process was carried out using Agilent Technologies' LC-20AD (LC 20AD from Japan) platform. The system included a PDA detector (G7115A), an auto-injector (G7129A), and a quaternary pump with a degasser (G7111A)—different data interpretation and analysis methods were used inside the sophisticated Open Lab platform. The high-performance chromatographic separation was carried out with a very good Phenomenex Luna C18 analytical column C-18(2) 100 with internal diameters of 250, length of 4.6mm, and 5µm particle size. This column from Phenomenex Inc., California USA, is made to ensure high accuracy in the chromatographic analysis.[10]

Selection of the mobile phase

To measure the quantity of Azelaic Acid, a variety of mobile phase ratios were tested. Taking into account system suitability parameters such as RT, tailing factor, number of theoretical plates, and HETP, the mobile phase found to be most suitable for analysis was 0.5% formic acid: acetonitrile in a 50:50 ratio for the density of vol (S), which can be run under isocratic conditions. The mobile phase was filtered using Whatman 0.45 µ filter paper and degassed by sonication. The flow rate employed for the analysis was 1 mL/min.

Preparation of Standard Stock Solutions

To make a standard stock solution, 10mg of Azelaic Acid was weighed and transferred into a 10ml volumetric flask. The substance was then dissolved in methanol and further diluted to reach a concentration of 1000 µg/mL.

Preparation of working standard Solutions

0.1ml of the solution was aliquoted from the Standard Stock Solution of Azeliac acid, transferred to 10 ml volumetric, and diluted up to 10 ml using the 50:50 To optimise HPLC analysis, combine 0.5% formic acid with 100ml of acetonitrile to get a solution concentration of 10 µg/ml.

Design of Experiment

We explored the isocratic elution optimisation program utilising a systematic experimental design method called Design of Experiments (DoE) and Response Surface Methodology (RSM). Specifically, in the current investigation, the experiment approach BBD(BOX- BEHNKEN DESIGN) with three factors and three centre points was applied, resulting in a total of fifteen experimental runs[11].Our research concentrated on optimising three essential independent variables: organic phase percentage (X1), flow rate ml/min (X2), and column temperature (X3), whereas the dependent variables were retention time (Y1), tailing factor (Y2), and theoretical plate (Y3). For DoE, the independent variables, organic phase percentage (X1), flow rate (X2), and column temperature with specified levels (-1,0,+1) are depicted in **Table 1**

Table 1. Chromatographic factors and level of BBD

Level	Organic phase %	Flow rate ml/min	Temperature
Low (-1)	40	0.8	25
Medium(0)	50	1	30
High (+1)	60	1.8	35

Analytical Method Validation

The new HPLC method was validated using ICH Q2 (R1) guidelines, which provided critical and additional parameters that improved the method's reliability and accuracy. These parameters included: defining LOQ and LOD for sensitivity analysis, checking linearity for the concentration-response relationship, estimating precision for repeatability and reproducibility studies, investigating robustness and ruggedness considerations, and finally testing accuracy for closeness to true values[12].

Calibration Curve and Linearity

Calibration of Azelaic Acid was performed at concentrations of 2, 4, 6, 8, and 10µg/mL. Each answer was prepared in three separate sets. To create the calibration curve, the height of the peak was used to measure the dependent variable, while the concentration of the substance was utilised as the independent variable. This was done after testing linearity over the same concentration range for three days.

System Suitability

To ascertain the reliability of the selected chromatographic system, quantitative analysis of peak area, TP, and TF for Azelaic Acid was carried out for six times.

Accuracy

Accuracy can therefore be defined as the comparison between the observed values resulting from the method and the true concentrations. The accuracy of the method was checked by spiking the drug-to-drug at three different levels of concentration 50% 100% and 150% of Azelaic Acid standard solution[13].

Precision

The repeatability and intermediate precision data were analysed routinely to determine the method's intra-day precision or repeatability, as well as its inter-day precision. The results of injecting replicate standard or sample solutions on the same and different days were compared, and the %RSD was calculated to show that the method's precision was adequate[14].

LOD and LOQ

Limit of Detection (LOD) and Limit of Quantification (LOQ) are measures of the smallest quantity of analyte or substance that a particular technology can distinguish from others. The calibration curve was used to calculate the LOD and LOQ. The LOD was defined as the smallest amount of analyte that elicited a response three times the standard deviation of the calibration curve's intercept. The LOQ was calculated by multiplying the calibration curve's intercept by 10 times its standard deviation[15].

Robustness

The reproducibility of the method is studied to find out its stability under slight variations in phases of the experiment. In the current study, changes made in the mobile phase composition and the flow rate of the mobile phase were used to define the robustness.

Ruggedness

Ruggedness was conducted to understand how the method performs when implemented by different individuals so as to keep the other variables affecting the experiments constant[16]**.**

Formulation and Evaluation of Azelaic acid loaded cubosomes

The top-down method was used for the preparation of cubosomes in which a mixture of Lipid and stabilizer was used. Glyceryl monooleate and poloxamer 407 were mixed and melted in a water bath at

60oC. Later on, 10mg of Azelaic acid was added to this mixture and stirred to dissolve. Then a drop by drop addition of preheated distilled water was made to the solution, and stirred consistently until it was added in completely. The solution was then left to equilibrate for one day to produce a two phase system that was disrupted after stirring. The whole system was homogenized at 15000 rpm for 10 min at room temperature and probe sonicator at 5 min. From such a liquid dispersion of cubosomes, the obtained product is kept at room temperature and in the darkness (without being exposed to direct sunlight).The structural analysis of synthesized Cubosomes included entrapment efficiency measurements, zeta potential, Particle Size, and transmission electron microscopy evaluation.[17]

3. Results and discussion

The BBD was utilized for optimization, selecting three independent variables from the factor screening studies to be adjusted at three levels: these are low, (-1), moderate, (0) and high (1). It has been found that they were the % of the Organic Phase, the Flow Rate and also the Column Temperature. The dependent factors that was captured in the chosen response variables had ; (Y1) RT, (Y2) TF, and (Y3) TP. The design response table of 15 runs using Box-Behnken Design is presented in Table 2. The retention time (Y1) responses recording for Azelaic Acid when recorded in minutes were noted to range from 2.13 to 3.29 minutes for the least and at the most respectively. Azelaic Acid was found to have a tailing factor (Y2) ranging between 1and 2.34. The responses of the Theoretical Plate (Y3) for Azelaic Acid ranged between 1923.21and 3102.45[18].

Table 2. Experimental layout, Factors, and observed outcomes by employing response surface methodology (Box-Behnken design) for the optimization.

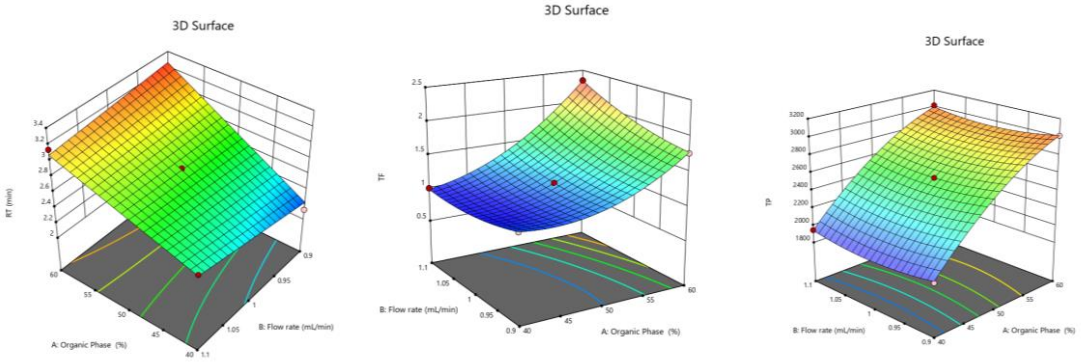
Sr.no	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
	A:Organic phase %	B:Flow rate	C:Temperature	RT	TF	TP
	%	mL/min		min		
1	40	1.1	30	2.55	1	1945.23
2	40	0.9	30	2.13	1.11	1923.21
3	40	1	25	2.47	1.14	1998.12
4	50	1.1	25	2.69	1.32	2684.23
5	50	0.9	35	2.68	1.41	2745.56
6	60	0.9	30	3.21	1.76	3014.21
7	60	1	25	3.29	2.14	2987.56
8	50	1.1	35	2.74	1.81	2749.54
9	40	1	35	2.45	1.11	1987.25
10	50	1	30	2.72	1.21	2547.23
11	50	1	30	2.79	1.21	2547.23
12	60	1.1	30	3.14	2.34	3014.25
13	50	0.9	25	2.87	1.32	2745.89
14	50	1	30	2.80	1.21	2547.23

15	60	1	35	3.1	2.14	3102.45

In software, ANOVA is used in the analysis of experimental outcome to determine the relevance of factors and interrelations where it comes to response variables. The results of the applied design are presented in the form of statistical information in Tables S1–S3. The polynomial equations use are in analyzing relationships between factors and response measures. As shown above the arrows, positive values will be assigned to capture the synergy influence while negative one will represent an antagonistic effect on the model. The observed high correlation coefficients of all the equations guarantee the achievement of the optimal values of the chosen responses (Table 2)[19].

Examination of the 3D response surface plots for Y1 (RT) shows that an increase in the organic phase results in a decrease in RT and increase in the flow rate leads to an increase in RT. It has been observed that slight variations of the column temperature do not have much impact in relation to the RT, which decreases with an increase in this temperature parameter (figure 2a). Similarly when increasing the flow rate for Y2 the concentration of the organic phase, the concentration of the tailing factor gradually increases and hence we agree that there is a direct relationship as portrayed by Figure 2b. For the purpose of comparison it is seen that there was no change in the tailing factor even if the column temperature was different[20]. On balance, for Y3 (theoretical plates), the enhancement of the Organic Phase Concentration and the Flow Rate as much as 50 %v/v and 1mL/min, respectively has resulted in higher theoretical plates, thus better separation. However, for these levels and beyond there is the decrease in the number of the theoretical plates (Figure 2c). Y3, the least sensitive to column temperature variation histograms shows a change of the column temperature even though a large value of column temperature is set as a default for Y3. Using Design-Expert software version 13.0 by Stat-Ease, Inc., optimal values for the parameters were identified: An organic phase of 50% v/v, a flow rate of 1 mL/min and a column temperature of 30◦C were used. These predictions may be regarded as fairly accurate as compared to the experimental outcome especially in Run 5 with a prediction error of 5%[21].

Figure 1. 2a :(Y1) Retention Time, 2b :(Y2) Tailing Factor, 2c:(Y3) Theoretical Plate



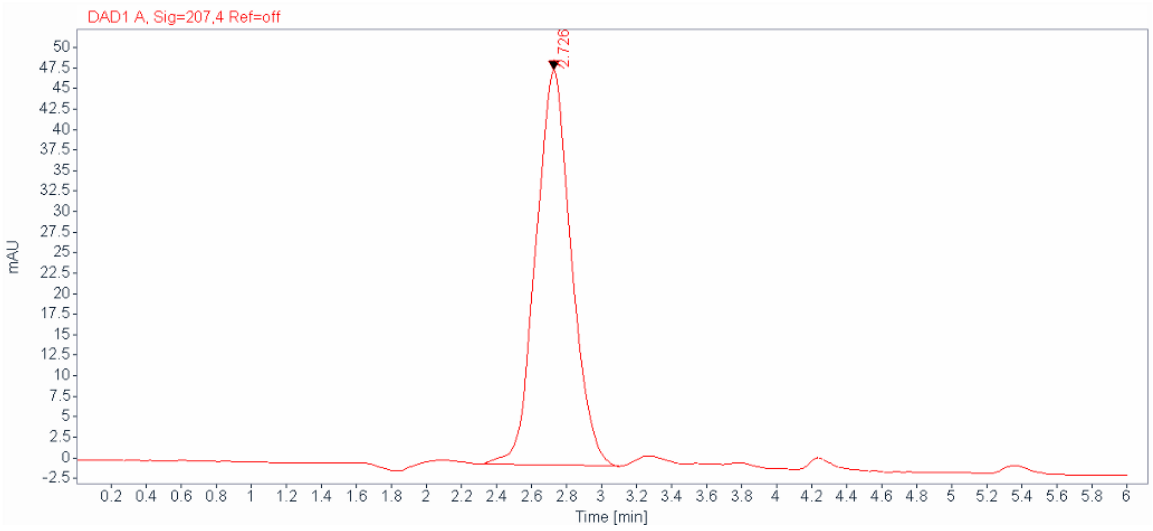
4. Analytical Method Validation

The validation of the optimized HPLC method for the estimation of Azelaic Acid was also followed and these parameters were found to be well within the prescribed limits of ICH Q2 (R1) Guidelines[22].

5. Method Development

Different mobile phases have been investigated to help develop an HPLC method for the determination of Azeliac acid. These include Methanol (v/v), 0.5Formic Acid-water, 30:70 (v/v), Methanol-Orthophosphoric acid (v/v), Methanol – Pure Water 30:70% (v/v) and Acetonitrile: 0.5 ml Formic Acid in MilliQ Water 60:40 v / v and Acetonitrile: 0.5 ml Ortho Phosphoric acid in MilliQ Water 30:70 (v/v). The chemical suitability of the mobile phase was judged based on the sensitivity of the assay, the phase for stability tests, ease of preparation, and the use of routine mobile phase solvents. A validated and selective RP-HPLC method was accurately established for the determination of Azelaic Acid with reasonable sensitivity and specificity. Following optimization, a mobile phase consisting of a 50:50ratio of 0.5% formic acid: Acetonitrile which gave good peak shape and efficient resolution of Azelaic Acid with a flow rate of 1ml/min and acceptable back pressure and at retention time of 2.76 min. The detection wavelength of 210 nm was set based on the absorbance maximum value of Azeliac acid[23]**.** The Standard Chromatogram of Azelaic Acid is shown in **Figure No** 3

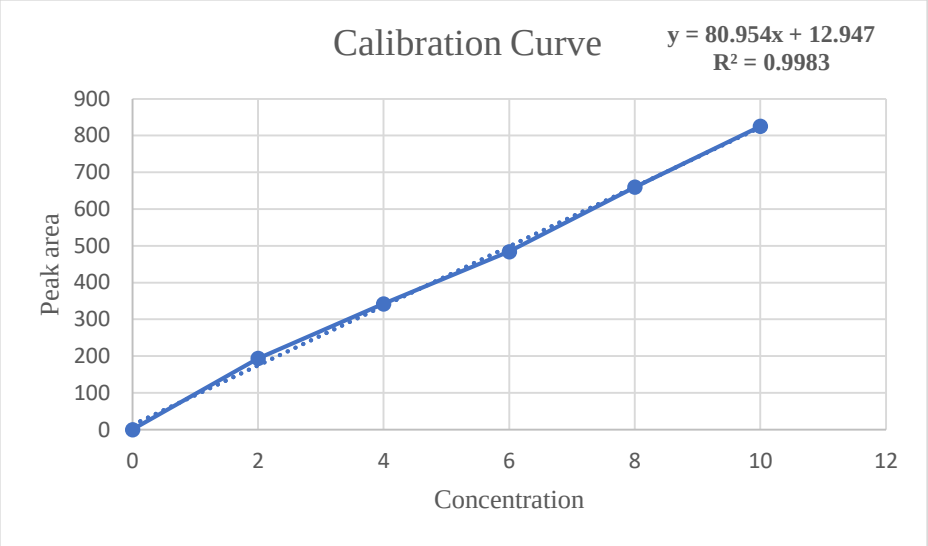
Figure 2. Standard Chromatogram of Azelaic acid



Calibration Curve and Linearity

The assessment of the linearity parameter was carried out at 5 different concentrations for the Azelaic Acid analyte. The concentrations of Azelaic Acid with the range of 2-10µg/ml were plotted. The plots of concentration against the peak area of calibration curves are presented in Figure 3. The obtained coefficient values were 0.995% for Azelaic Acid which shows good linearity for the concentration range of Azelaic Acid above. The calibration curve of concentration and peak area is illustrated in Figure 4 to plot concentration against peak area. The present correlation coefficients obtained were 0.995 % for Azelaic Acid, which showed very good linearity in terms of concentration within the studied concentration range. This assessment maintains the accuracy of the method in estimating the concentration of an analyte within the stated range which makes the method more reliable and suitable within the analyses.

Figure :3 CALIBRATION CURVE OF AZELAIC ACID



System Suitability

To ascertain the reliability of the selected chromatographic system, quantitative analysis of peak area, TP, and TF for Azelaic Acid was carried out for six times And the results are presented in Table 4.

Table 3. Parameters of System Suitability

SR.NO	System Suitability	Azelaic Acid	
		Mean ±SD	%RSD
1	Peak	220.09±0.150	0.135
2	Retention Time	2.76±0.005	0.074
3	Theoretical Plate	2547.23±0.351	0.005
4	Tailing factor	1.21±0.005	0.215

Accuracy

The results were therefore computed and presented in Table 5 and the value met the acceptable percentage recovery requisite. This reaffirms the ability of the method to estimate solutions that provide a nearly true solution to the problem thus confirming the efficiency of the method that can be measured quantitatively.

Table 4. Accuracy

Accuracy%	Theoretical (µg/mL)	Actual (µg/mL)	%Recovery
50%	15	14.935	99.57%
100%	20	19.450	97.25%
150%	25	24.965	99.86%

Precision

In these studies, the outcomes were stated as %RSD, and the qualities had been obtained beneath 2%, as a result in Table 6 illustrates the fact that this specifically prepared HPLC method has increased accurate and precise outcomes. This confirms the reliability of the method in the determination of Azelaic Acid at varying experimental conditions and time**.**

Table 5. Intraday and Interday

Concentration (µg/mL)	Intraday	Interday
2		
Mean ±SD	220.52 ±0.47	221.90±0.40
%RSD	0.216%	0.225%
6		
Mean ±SD	484.29±0.49	485.19±0.45
%RSD	0.149%	0.150%
10		
Mean ±SD	658.55±1.20	658.99±1.55
%RSD	0.183%	0.185%

LOD and LOQ

In the present investigation, LOD and LOQ value for Azelaic Acid was evaluated and presented as follows in Table 7. These LOD and LOQ results of this work show that the presented method is efficient in detecting Azelaic Acid with low sensitivity interference disturbances as described in this work are perfect for quantification of with high accuracy and minimal detectability analytical application.

Table 6. LOD and LOQ

Drug	LOD	LOQ
Azelaic Acid	1.521 µg/mL	3.342 µg/mL

Robustness

The notation of the percent relative standard deviation %RSD computed is smaller and is below 2% as contradicted by the results presented in Table 8 as showing that the method had a satisfactory reproducibility even if marginally different values of these parameters were used. This demonstrates the effectiveness of the method and how outcomes could be obtained irrespective of the approach that may be used.

Table 7. Robustness

Conditions Changed	Changes Mode	AUC± SD	%RSD
Flow rate (mL/min)	0.9ml/min	205.38±1.9	0.450%
	1.1ml/min	224.36±2.48	0.940%
Wavelength (nm)	208	205.6±0.15	0.076%
	212	222.4±0.26	0.129%
Mobile phase ratio (v/v)	55:45	210.83±0.05	0.029%
	45:55	215.6±0.43	0.212%

Ruggedness

In this analysis, it was shown that all the values of %RSD are less than 2% and tabulated as shown in Table 9 below. As this implies, there was no inter-operator variability with regard to the precision and repeatability of the method.

Table 8. Ruggedness

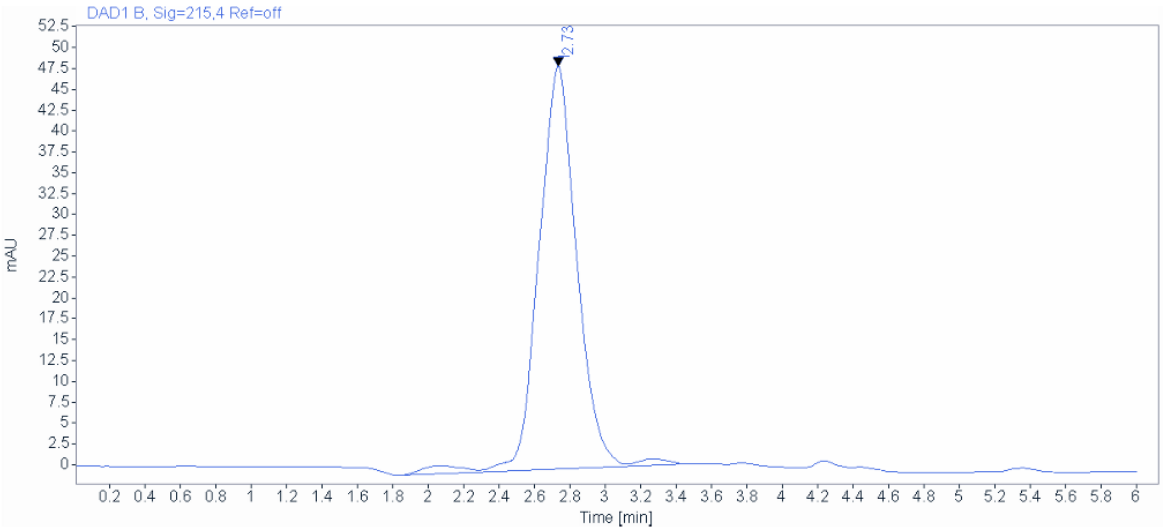
Concentration (µg/mL)	Analyst 1	Analyst 2
15	484.35	485.09
	486.54	484.36
	482.26	486.25
	488.63	489.26
	483.38	480.62
	480.89	482.49
Mean	484.33	484.67
Standard Deviation	1.55	1.99

% RSD	0.587%	0.618%

Formulation:

The optimized method was successfully used for the estimation of Azelaic Acid in the Azelaic Acid Loaded Cubosomes formulated in this study through the quantitative analysis of data obtained and peak area and sensitivity, accuracy and percentage coefficients of variation revealed adequate and ideal outcomes. In this work, other studies justifying the possibility of using this method in routine analysis have been presented. The HPLC chromatogram of the prepared Almotriptan Malate-loaded Cubosomes is depicted as in **figure 6.**

Figure 4.Chromatogram of Formulation



Evaluation of the Azelaic Acid loaded Cubosomes

The prepared Azelaic Acid -loaded Cubosomes achieved a Particle size **(fig.5a)**measurement of 185 mV while exhibiting a Zeta Potential Value of -14.36 **(Fig.5b)**. Research indicates that ZP recognizes and evaluates niosome particle size distribution by providing values below one, indicating proper dimension control. Experimental results showed a 92.4% success rate for medicine encapsulation within nanoparticles. The high entrapment efficiency value indicates reduced drug loss during fabrication, thus offering a promising pharmacological outcome. The Transmission electron microscopy pictures showed spherical Cubosomes forming particles with a smooth surface appearance, as displayed in **Fig.6**. According to this observation, a successfully synthesized nanoparticle demonstrates its presence through consistent, well-defined morphology[24].

Figure 5a: Particle Size **Figure 5b : Zeta Potential**

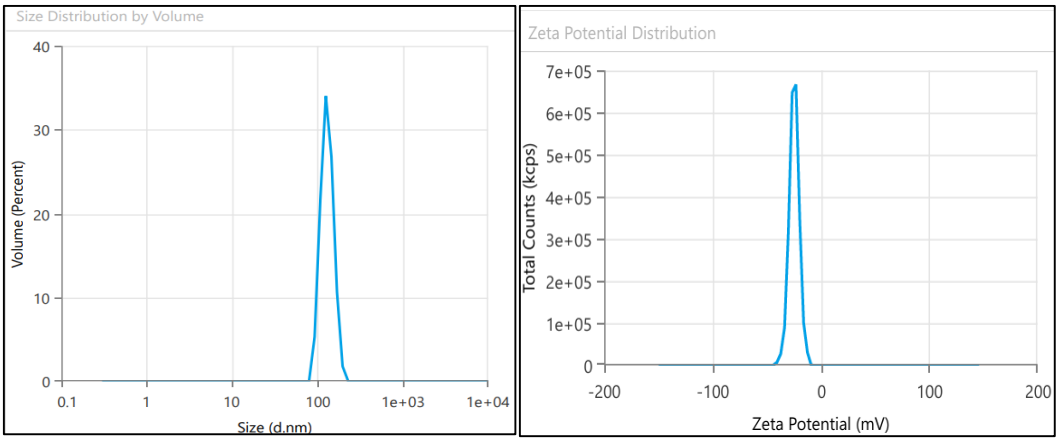
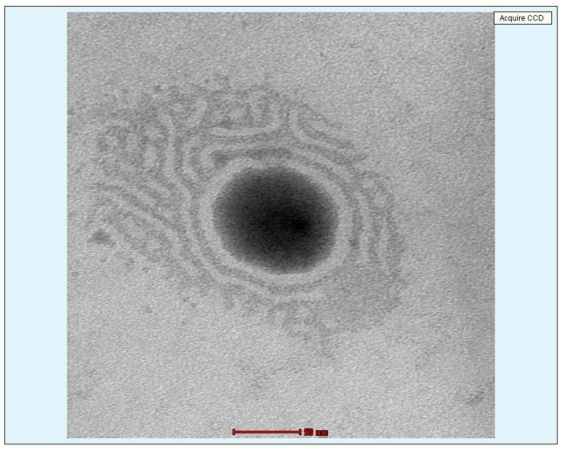


Figure 6 : Tem analysis of Formulation



6. Conclusion :

This study effectively created and verified a reliable and accurate RP-HPLC technique for measuring azelaic acid in pharmaceutical formulations and bulk. Box-Behnken Design was used to optimise the procedure, and ICH Q2 (R1) guidelines were followed for validation. Its capacity to indicate stability was confirmed by its outstanding linearity, accuracy, precision, and sensitivity, as well as its ability to separate Azelaic Acid from its breakdown products under a variety of stress setting. Additionally, a top-down method was successfully used to create cubosomes laden with azelaic acid. These cubosomes' aptitude for improved drug administration was confirmed by their steady zeta potential, ideal particle size, and good entrapment efficiency. Azelaic Acid's solubility and stability were enhanced by the formulation, suggesting that it might be applied topically with success. All things considered, the technique provides a dependable, and repeatable method for regular formulation analysis and quality control of azelaic acid. Its promise as a useful tool in pharmaceutical research and development, especially for dermatological medicines utilising azelaic acid, is highlighted by its effective implementation in both formulation studies and analytical validation.

Declarations

This research has no conflict of interest among the authors according to the disclosure statement.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Data availability

The authors have all endorsed their involvement in both the research design and implementation phase of this academic paper. All authors have thoroughly evaluated the research paper while maintaining its approval status during manuscript development stages.

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List of Abbreviations

AA: Azeliac acid **,ICH:** International Conference of Harmonisation**, QbD:** Quality by Design**, HPLC:** High-Performance Liquid Chromatography**, DoE:** Design Of Experiment, **RSM:** Response Surface Methodology, **BBD:** Box-Behnken Design, **LOD:** Limit of Detection, **LOQ:** Limit of Quantification.

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