RESEARCH PAPER

Validated Reverse Phase High Performance Liquid Chromatography Approach via Experimental Design for Accurate Embelin Estimation in Pharmaceutical Products

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ABSTRACT

Objective: A Box-Behnken experimental design was employed within a quality by design approach (QbD) to develop a rapid and straightforward Reverse-phase high performance liquid chromatography (RP-HPLC) method for quantifying embelin (EMB) in bulk drug and formulations requiring fewer runs for optimization.

Method: Independent variables - flow rate (A), organic solvent ratio (B), and column temperature (C) were optimized with respect to response variables: retention time (Y1), and % recovery (Y2). Statistical significance was confirmed via analysis of variance (ANOVA). Chromatographic separation used a C18 column (250 × 4.6 mm, 5 µm particle size) with an isocratic mobile phase (90:10 v/v) of 0.1% phosphoric acid in methanol (A) and 0.1% phosphoric acid in water (B) at flow rates of 0.6–1.4 ml/min. The method proved simple, sensitive, and precise for routine EMB quantification.

Results: The method demonstrated excellent linearity ($R^2 > 0.999$, 125−750 µg/ml), accuracy as (% recovery) 99.146-100.691%, limit of detection (LOD) (0.06825 µg/ml), and limit of quantification (LOQ) (0.20683 µg/ml). It was successfully applied for EMB quantification in nano-formulation.

Conclusion: All validation parameters (linearity, accuracy, recovery, LOD, and LOQ) met acceptance criteria, affirming that RP-HPLC method could be suitably employed for EMB estimation in the bulk drug and nano-formulations.

Keywords: high performance liquid chromatography (HPLC), quality based design (QbD), box Behnken experimental design (BBD).

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INTRODUCTION

Phytoconstituents like Embelin (EMB), a natural benzoquinone as potential therapeutic agents have gained significant attention due to their wide range of pharmacological activities and relatively low toxicity [1]. To effectively harness its therapeutic potential, EMB is increasingly being incorporated into advanced drug delivery systems including nano-formulations, with aim of improving its bioavailability, stability and targeted delivery. Therefore, the accurate quantification of EMB in both bulk drug and complex formulations matrices is essential for quality control, and formulation development.

Several analytical methods, including spectrophotometric, and chromatographic technique have been reported for EMB estimation. Although, HPLC is considered as a preferred method due to its

sensitivity and reproducibility. From the literature background, analytical method for EMB estimation reported long run times, complex mobile phase compositions, poor sensitivity, and validation parameters with respect to robustness and applicability to nano-formulations [2,3]. Furthermore, most of the literature are restricted to bulk drug analysis or plant extracts and limited or no uses with lipid-based delivery systems, where excipient interference and matrix effects can significantly influence analytical performance. These shortcomings point to unmet gap in the literature for a simple, rapid, sensitive, and robust HPLC method applicable to both bulk EMB and nano-formulation [4,5].

EMB, (2,5 dihydroxy-3-undecyl-1,4 benzoquinone) is the principal bioactive constituent of *Embelica ribes* fruits (Figure 1) present at a concentration ranging from approximately 2.3% to 4.33% [6].

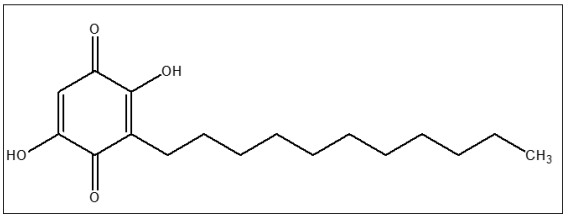

Figure 1. The structure of the EMB

In view of the therapeutic relevance of EMB and the shortcomings of existing analytical methods, the present study aims to develop and validate a novel, optimized, and robust hplc method for the quantification of EMB in both bulk drug and lipid-based nano-formulations. Furthermore, a Design of Experiments (DoE) approach has been employed to systematically optimize chromatographic conditions. Compared to the conventional One Variable at a Time (ovat) approach, DoE enables the evaluation of interactions between critical method parameters with significantly fewer experimental runs, resulting in reduced time, cost, and enhanced method understanding [7]. The developed method is specifically designed to be sensitive, reproducible, and applicable to nano-formulation matrices, thereby addressing an unmet analytical need in current EMB analysis.

MATERIAL AND METHODS

Chemicals and reagents

The EMB was supplied by Yucca Enterprises (Maharashtra, India). Water, orthophosphoric acid, and HPLC grade methanol were obtained from Laboratory chemicals. Throughout the investigation, only analytical-grade reagents were used.

Instrumentation and Chromatographic Conditions

The Shimadzu model HPLC system comprised a quaternary pump, a series of UV-Vis detectors column oven, a heated column thermostat, and a mobile phase degasser. The HPLC settings used for the optimization are as follows, based on earlier research: An Avantor ACE C18 HPLC column (250 mm × 4.6 mm, 5 µm particle size) was used as the stationary phase [8]. The mobile phase A was consisted of 0.1% phosphoric acid in methanol, and mobile phase B was consisted of 0.1% phosphoric acid in an aqueous solution, and both solutions were flowed in isocratic mode (90:10) at a 1 ml/min flow rate. The column temperature was maintained between 28 °C. The UV detection was carried out at 280 nm. A constant 30 µl injection volume was used. Before the drug solution was injected, the column was allowed to equilibrate up to sixty minutes while the mobile phase passed through the apparatus.

Preparation of Mobile Phase

Mobile phase A: A 1000 ml volumetric flask was partially filled with a precisely measured volume of methanol. Then, 1 ml of orthophosphoric acid was added, and the volume was made up to the mark with methanol. The resulting solution was filtered through a 0.45 µm membrane filter, and sonicated in a water bath for 10 minutes. After labelling, the solution was stored for further use.

Mobile Phase B: An accurately measured quantity of water was transferred into a 1000 ml of volumetric flask. Then, the volumetric flask was filled with 1 ml of orthophosphoric acid, and the final volume was made up to the mark with water. The resulting solution was filtered through a 0.45 µm membrane filter and sonicated in a water bath for 10 minutes. After labeling, the solution was stored for further use.

Preparation of Stock Solutions and working solution

Accurately weigh 10 mg of EMB and dissolved in 5 ml of methanol in a 10 ml volumetric flask. The mixture was sonicated for 1 minute to obtain a clear solution, then diluted with methanol to prepare a 1000 µg/ml standard solution. Working solutions were prepared by withdrawing accurately measured volumes of stock solution and transferring into separate 10 ml volumetric flask. Further, methanol was added with continuous shaking and diluting final volume up to the mark. Each solution was labeled and stored for future application. Solutions

were filtered through a 0.22 µm membrane filter before injecting into system, labeled, and stored.

Implementation of Quality by Design Avenue

QbD is a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management. It also includes designing tests and controls based on the scientific understanding established during the development stage.

In this study, a Box Behnken design was employed as a DoE to optimize the RP-HPLC method, with flow rate, organic phase, and column temperature as independent variables, and retention time and % recovery as response variables [9].

Method Optimization

A novel RP-HPLC technique was developed for EMB measurement using mobile phase of water, methanol, and orthophosphoric acid to ensure effective separation of bioactive components. The selection of the mobile phase was based on previous studies involving these solvents. Three independent variables were chosen for statistical analysis: flow rate (variable A), the organic phase ratio (variable B), and column temperature (variable C). Response variables included EMB recovery % (Y2) and retention time (Y1) which were used as robustness parameters. The method optimization was performed using response surface methodology, combining Design-Expert software and Box Behnken design (bbd). Seventeen experimental runs were conducted as per the design matrix's with results are shown in Tables 1 and 2. Analysis of variance (anova) was utilised to identify the significant differences across the design matrix. Response surface morphology was further used evaluate the effect of independent variables on the response, thereby determining the methods robustness and optimal mobile phase composition [10]. The Design Expert software suggested the 17 runs as shown in Table 2.

Table 1. Box–Behnken design: Independent variables: flow rate (A), organic phase ratio (B), column temperature (C), and response variables: retention length (Y1) and recovery % (Y2) with actual and coded levels.

Factor	**Name**	**Units**	**Low Actual**	**High Actual**	**Low Coded**	**High Coded**	**Mean**
A	Flow rate	ml/min	0.6	1.4	−1	+1	1
B	Organic phase ratio	-	85:15	95:5	−1	+1	90:10
C	Column temperature	°C	25	35	−1	+1	30
Response	Name	Units					
Y1	Retention time	min.					
Y2	% recovery	%					

Footnote: –1 corresponds to the lowest and +1 corresponds to highest experimental level of the independent variable.

Table 2 displays the 17 experimental runs that made up the design, which used the computer-generated quadratic model as follows:

$$Y = \beta 0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 AB + \beta_5 AC + \beta_6 BC + \beta_7 A^2 + \beta_8 B^2 + \beta_9 C^2$$

Where A, B, and C are the levels of independent variables, β0 is a constant, β1, β2, and β3 are linear coefficients, β4, β5, and β6 are interaction coefficients among three components, and β7, β8, and β9 are quadratic coefficients of observed experimental values. The measured response for each combination of factor levels is denoted by Y. The fit of the model was verified using the coefficient of determination (R^2) obtained through anova. The statistical significance of the model was confirmed by the F-test and P-value. The effectiveness of the fitted model for the responses was evaluated using anova. Additionally, the method was further validated using software analysis based on the "Desirability function" criteria [11-13].

Validation of Analytical Method

Several parameters, including the system suitability test, linearity, specificity, accuracy, precision, limit of detection (LOQ), and limit of quantification (LOQ), were assessed to validate the proposed method in accordance with the ICH Q2(R1) guidelines published in 2005 [14].

METHOD VALIDATION

Optimized chromatographic conditions

The Shimadzu HPLC system consisted of a quaternary pump, a series of UV-VIS detectors a column oven with a heated thermostat, and a mobile phase degasser. The system was optimized using conditions based on prior research. Separation was performed on an Avantor ACE C18 HPLC column (250 mm × 4.6 mm, 5 µm particle size) used as stationary phase. The mobile phase comprised 0.1% phosphoric acid in methanol (mobile phase A) was, and 0.1% phosphoric acid in water (mobile phase B) delivered in an isocratic mode with a ratio of 90:10 (A: B) at a flow rate of 1 ml/min. The column temperature was maintained between 28 °C. UV detection was carried out at 280 nm, with a consistent injection volume of 30 µL. Before the sample injection, the column was allowed to equilibrate up to 60 minutes by passing the mobile phase through the system.

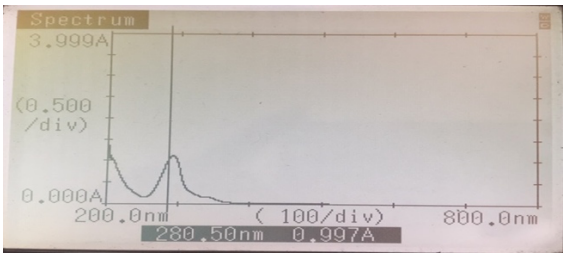

Figure 2. UV absorption spectrum of EMB standard (10 µg/ml) in methanol showing λmax at 280 nm.

System suitability

Six replicate injection of the 500 µg/ml embelin working standard were analysed HPLC to evaluate system suitability. The acceptability criteria for requirements for retention time and tailing factor were investigated based on these injections.

Preparation: Accurately weighed 10 mg of embelin was transferred into a 10 ml volumetric flasks, dissolved in methanol. The solution was sonicated for 1min, to ensure complete dissolution, yielding a standard stock solution of 1000 µg/ml. From this standard stock, a working solutions of 500 µg/ml were prepared by withdrawing 5 ml of the stock solution into a 10 ml volumetric flask and diluting to volume with methanol. The resulting solution was filtered through a 0.22 µm membrane filter paper. The filtered sample was used for HPLC analysis. The chromatograms were recorded to determine the determine the peak area and retention time of each working solution. The %RSD for the peak area was determined [15].

Specificity

Specificity is a critical parameter in hplc analysis, reflecting the the analytical system's capacity to identify the analyte and without interference with from other components. To determine specificity, chromatograms of the blank solution (mobile phase and solvent), the placebo, and their combination were analysed separately at middle quality control level (500 µg/mL). This assessment ensures that no interference from solvent or excipients at retention time of analyte.

Linearity

A calibration curve in the concentration range of 125-750 µg/ml was prepared using six serial dilutions of standard EMB. The linearity of the method was confirmed by coefficient of determination value (R^2) obtained from regression equation which showed consistent detector response across all concentrations. Graphing the concentration of the standard solution on the X-axis and the peak area on the Y-axis were made to obtained the calibration curve [15].

Preparation:

From 1000 µg/mL stock, dilute 1.25, 2.5, 3.75, 5.0, 6.25, 7.5 mL to 10 mL methanol for 125, 250, 375, 500, 625, 750 µg/mL working standard, filtered through 0.22 µm membrane and injected into the HPLC system. The peak area of embelin was recorded for each working solution, and a calibration curve was constructed by plotting concentration (X-axis) against peak area (Y-axis).

LOQ and LOD

The LOD refers to the minimum quantity of analyte in a sample that can be identified but not necessarily quantified with accuracy or precision. The LOQ is the minimum amount that can be quantitatively measured with the acceptable precision and accuracy. The LOQ and LOD calculated via signal-to-noise ratio or standard deviation (σ) of responses and slop (S) using equations 1 and 2.

$$LOD = 3.3 \times \frac{\sigma}{S} \ldots\ldots\ldots\ldots \text{Equation 1}$$

$$LOQ = 10 \times \frac{\sigma}{S} \ldots\ldots\ldots\ldots \text{Equation 2}$$

Accuracy (% Recovery)

A 500 µg/ml of the sample was pre-quantified, and spiked with 75% (3.75 ml stock), 100% (5 ml stock) or 125% (6.25 ml stock) levels in separate 10 ml flasks containing 5 ml sample. Each sample was diluted to the mark with methanol, filtered, and injected into the HPLC system.

Chromatograms were recorded, peak areas were measured, % recovery, % RSD, and mean recovery

were calculated to validate accuracy, ensuring accurate measurement of analyte in the sample.

Precision and repeatability

As per ICH guidelines, precision, includes both intra-day and inter-day variation. A stock solution of 1000 µg/ml was prepared by dissolving 10 mg EMB in 10 ml methanol and sonicating until clear. Working solutions of 375, 500, 625 µg/ml were prepared by appropriate dilution with methanol, filtered through a 0.22 µm nylon membrane, and analyzed by RP-HPLC.

For intra-day precision (repeatability), six replicates of each concentration were injected on the same day. For intermediate precision (inter-day precision), six replicates of each concentration were analyzed over six consecutive days under same environmental conditions. The resulting %RSD value were used to assessed precision and repeatability [16].

Ruggedness and Robustness

Ruggedness was assessed by having two independent analysts analyze the Embelin solution under same condition. For each chromatographic peak, the % recovery and % RSD were calculated from the corresponding peak area.

Robustness of the HPLC method was assessed by deliberately varying chromatographic conditions. The method was considered robust if the results remain within acceptance limits. The tested parameters included flow rate (± 0.1 ml/min) and the organic phase ratio, as both can influence retention time and % recovery [17].

For this study, the previously prepared 500 µg/mL embelin working solution was filtered through a 0.22 µm nylon membrane filter and injected into the HPLC system for chromatographic analysis. The peak area and retention behavior were recorded for evaluation [18].

Sample preparation and determination of entrapment efficiency

EMB-loaded glycerosomes (GLSMs) were developed using the modified thin-film hydration method (Bangham method). EMB was dissolved in chloroform: methanol (7:3 v/v), sonicated (1 min), and transferred to a 100 ml round-bottom flask. Organic solvents were evaporated using a rotary evaporator (60°C, 50 rpm, reduced pressure) to form a thin lipid film, which was dried in a desiccator. The film was hydrated in two stages with 10 ml glycerol-water solution: first with 5 ml (60°C, 1 h rotation), then with remaining 5 ml (60°C, 1 h rotation). The dispersion was probe-sonicated (40% amplitude, 25 cycles: 4 s on/5 s off).

Entrapment efficiency (EE%). The GLSMs suspension was centrifuged (15,000 rpm, 4 °C, 20 min). The supernatant was discarded, and the pellet was washed with Milli-Q water, reconstituted in methanol, and analyzed by the validated RP-HPLC method using the standard calibration curve.

EE% = [(Total drug - Free drug)/Total drug] × 100

Results and Discussion Method Development

Preliminary experiments based on a literature search and trial-and-error approach were conducted to optimize a straightforward, economical, accurate, and precise method for evaluating EMB in their bulk forms and for quality control. The first step in the development process was selection of mobile phase from various solvent combination including acetonitrile, tetrahydrofuran, methanol, and o-phosphoric acid.

Optimization using the Box Behnken design

The Box Behnken design was selected for HPLC method optimization due to its ability to generate second-order equations relating independent variables to responses with fewer experimental runs compared to central composite design (cdd). Unlike cdd, bbd avoids extreme experimental conditions (±α levels) that may yield unreliable results. In this study, bbd was employed to optimize retention time (Y1, min) and % recovery (Y2) for optimal EMB separation achieving minimum retention time and maximum recovery. Three independent variables; flow rate, organic phase ratio, and column temperature at three levels were used to design the bbd design matrix, yielding a total of 17 runs as shown in Table 2 [19].

Table 2. Box–Behnken design matrix for 17 experimental runs showing independent variables flow rate (A), organic phase ratio (B), column temperature (C)) and responses, retention time (Y1), % recovery (Y2).

Run	Factor 1 A: Flow rate (ml/min)	Factor 2 B: Organic phase ratio	Factor 3 C: Column temperature (°C)	Response 1 Retention time (min.)	Response 2 % recovery (%)
1	1.4	90:10	30	6.78	90.32
2	1	95:5	30	9.1	99.69

3	0.6	90:10	35	15.95	88.12
4	1.4	90:10	25	7.2	85.14
5	1	85:15	25	9.71	92.14
6	1	95:5	25	9.63	75.12
7	0.6	90:10	25	16.3	93.11
8	0.6	90:10	30	16.14	78.15
9	1	90:10	30	9.46	99.85
10	1	90:10	30	9.4	99.63
11	1	85:15	35	9.35	78.05
12	1	95:5	30	9.46	99.89
13	1	85:5	30	9.45	99.78
14	0.6	90:10	30	16.08	90.10
15	1.4	85:5	35	6.47	91.10
16	1	90:10	35	9.01	90.15
17	1.4	95:5	30	6.98	73.13

Factor	**Name**	**Units**	**Low Actual**	**High Actual**	**Low Coded**	**High Coded**	**Mean**
A	Flow rate	ml/min	0.6	1.4	−1	+1	1
B	Organic solvent ratio	-	85:15	95:5	−1	+1	90:10
C	Column temperature	°C	25	35	−1	+1	30
Resp	Name	Units					
on se							
Y1	Retention time	min.					
Y2	% recovery	%					

Retention time

The three independent variables were used to optimize the retention time. All trial results were analysed by anova for the quadratic model, as presented in Table 3, indicating the robustness of the bbd response surface. The model is highly significant, with an F-value of 1177.33 and only a 0.01% probability that this result is due to noise. Model terms with 'Prob > F' values below 0.0500 are considered significant. In this case, factors A, B, C; their interaction term AB, and quadratic terms A^2 are significant. The Lack of Fit F-value of 0.62 compared to pure error indicates a non-significant lack of fit, with a 97.73% probability this is due to noise, which is desirable for model compatibility.

Both Adjusted R-Squared (0.9985) and Predicted R-Squared (0.9967) are close to 1, indicating an excellent fit of the model. Adequate Precision measures the signal-to-noise ratio, and value greater than 4 is desirable. This model demonstrates an adequate signal and is suitable for navigating the design space. Using four variables, the quadratic model was found to be the most effective for fitting the data. The polynomial equations for the response factors are as follows:

Retention time: $9.38 - 4.66 \times A - 0.1491 \times B - 0.2725 \times C + 0.3023 \times A \times B - 0.1030 \times A \times C - 0.1620 \times B \times C + 2.08 \times A^2 + 0.0232 \times B^2 - 0.0100 \times C^2$

Table 3. Analysis of Variance for the method.

Source	**F Value**	**p-value**	**Prob > F**
Model	1171.33	< 0.0001	Significant
A-Flow rate	7969.54	< 0.0001	
B-Organic phase ratio	5.86	< 0.0460	
C-Column temperature	23.80	< 0.0018	
AB	226.117	0.049	

AC	2954.640	0.23	
BC	2157.432	0.12	
A^2	864156.502	< 0.0001	
B^2	8.426	0.79	
C^2	300.151	0.90	
Lack of Fit	0.062	0.67	Not significant
R-Squared	0.9993		
Adj R-Squared	0.9985		
Pred R-Squared	0.9967		
Adeq Precision	95.57		

It is evident that increasing the flow rate decreases retention time, while a reduce in phase ratio from mobile phase A to B slightly increase the retention time as seen Run 5, Run 11 and Run 13, respectively. The impact of column temperature and flow rate on the retention time were also assessed: retention time decreases drastically with higher flow rates, and it decreases with increasing column temperature. This may be due to reduce viscosity of mobile phase at higher temperature, which limits interaction time with stationary phase shorten retention time. Changes in organic phase ratio and column temperature were analysed; gradual increases in column temperature reduces retention time, whereas, alteration in organic phase ratio cause minimal change in retention time.

% recovery

The three independent factors were used to maximize the % recovery. Table 4 displays the ANOVA conclusion regarding the quadratic model, indicating that the BBD surface response was robust. The model's F-value of 111.33 signifies its statistical significance, with only a 0.01% probability that such a high F-value is due to noise. The model terms are considered significant when "Prob > F" is less than 0.0500.

In this instance, A, B, C, AC, AB, BC, A^2, B^2, and C^2 are significant model terms. There are non-significant model terms present when the values are greater than 0.1000. Model reduction may improve the model by removing superfluous terms, except those required to maintain hierarchy. The Lack of Fit F-value of 0.12 indicates that the lack of fit is not significant compared to pure error.

The signal-to-noise ratio exceeding 35.67 indicates a strong enough signal, making the model suitable for navigating the design space. Although a non-significant lack of fit is preferable, the high F-value suggests the model is statistically significant and robust, with only a 0.01% chance that noise is the source of this F-value. The model's Pred R-Squared and Adjusted R-Squared values are both 1.0000, indicating excellent agreement.

The 'Adequate Precision' value of 35.67 further confirms a sufficient signal-to-noise ratio, with values above 4 being considered desirable in chromatography modeling. The polynomial Equations for the response factors are given below:

% recovery: $9.38 - 4.66 \times A - 0.1491 \times B - 0.2725 \times C + 0.3023 \times A \times B + 0.1030 \times A \times C - 0.1620 \times B \times C + 2.08 \times A^2 + 0.0232 \times B^2 - 0.010 \times C^2$

Table 4. Analysis of variance for the % recovery.

Source	**F Value**	**p-value Prob > F**	
Model	111.33	< 0.0001	Significant
A-Flow rate	6.33	0.0401	
B-Organic phase ratio	91.73	< 0.0001	
C-Column temperature	16.22	0.005	
AB	427.08	< 0.0001	
AC	0.670	0.44	
BC	172.29	< 0.0001	
A^2	235.56	< 0.0001	
B^2	46.84	0.0002	

C^2	86.51	< 0.0001	
Lack of Fit	2.87	0.2064	Not significant
R-Squared	0.9931		
Adj R-Squared	0.9841		
Pred R-Squared	0.9067		
Adeq Precision	35.65		

The recovery % increases when the flow rate is increased from 0.9 to 1 ml/min, but further improvement leads to a decrease in percentage recovery. Likewise, increasing the organic phase ratio and column temperature increases the EMB's recovery% to a certain point. At a low flow rate of 0.6 ml/min, the recovery percentage is considerably low, likely due to the insufficient or slow elution of the analyte. The analyte may interact for extended time with the stationary phase or get adsorbed, resulting in lower recovery. Increasing the flow rate to 1.0 ml/min resulted in an improved recovery of 99.89% (Run 12), which is the highest among all runs. However, a higher flow rate of 1.4 ml/min negatively affected the recovery, as observed in Run 4 (73.13%) and Run 17 (85.14%). The mobile phase passes too quickly through the system at higher flow rate, giving the analyte less time to interact with stationary phase, this lead to poor elution and causing a drop in recovery.

Optimum condition

The flow rate of 1 ml/min, the organic phase ratio of 90:10 v/v, and a column temperature of 28 °C were identified as the optimal conditions for the independent variables. Under these condition, the predicted values for the response variables — retention time and % recovery, were 9.45 min and 98.95%, respectively. Experimental validation yielded retention time and % recovery of 9.47 min and 99.21% corresponding to percentage errors of 0.16% and 0.27% (Table 5). The chromatogram of EMB under these optimal conditions is shown in Figure 3, with no interfering peaks observed from the matrix. The retention time of EMB under these condition was 9.47 min**.** These optimized conditions were subsequently used for the system suitability test and validation procedure.

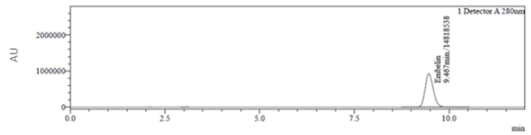

Figure 3. Representative HPLC chromatogram of the EMB working standard (250 µg/ml) obtained under optimized condition: flow rate 1 ml/min, organic phase ratio 90:10 v/v, and column temperature 28 °C.

Table 5. Estimated and predicted values of optimal HPLC Parameters with percentage error

Independent variables	Optimized formula	Response variables	Estimated values of responses	Predicted value of response	% Error†
Flow rate	1 ml/min	Retention time	9.47	9.45	0.16
Organic phase ratio	90:10	% recovery	99.21	98.95	0.27
Column temperature	28 °C	-	-	-	-

$$\dagger\%\text{Error} = \frac{(Predicted - Experimental)\ value}{Predicted\ value} \times 100$$

Validation of Optimized HPLC Method

Method validation is the quantitative procedure to confirm that an analytical test system is suitable for its intended purpose and capable of producing accurate results.

In our study, reasonable standard variations were fitted to relevant data models using the mean recovery approach, and the high-quality recovery values obtained confirmed statistical acceptability of the results. Consequently, our system was acknowledged as having statistical validity. Linearity, accuracy, precision, stability, robustness, and ruggedness were among the parameters that were later verified [20].

System suitability

The sample batch's system suitability test results for tailing factor and retention time were reproducible. The chromatographic system demonstrated good based on the standard deviation (SD) and % relative

standard deviation (% RSD) values. The % RSD for the tailing factor and EMB retention times was found to be 0.283% and 0.032%, respectively. All these results met with the acceptance criteria, where %RSD indicates < 5% Table 6 [21].

Table 6. System suitability factor†

Concentration (µg/ml)	Retention time (min.)	Tailing factor
500	9.467	1.175
500	9.462	1.170
500	9.458	1.172
500	9.463	1.179
500	9.465	1.177
500	9.462	1.173
Mean	9.463	1.174
STD	0.003	0.003
%RSD	0.032	0.283

†*Chromatographic condition as mentioned in the instrumentation and chromatographic condition under section 2.2.*

Specificity

The specificity was established by comparing the chromatograms of the blank solution with EMB standard solutions using a developed analytical method (Figure 4A−C). After injecting of 30 µL of analyte into the HPLC system, the chromatograms were examined.

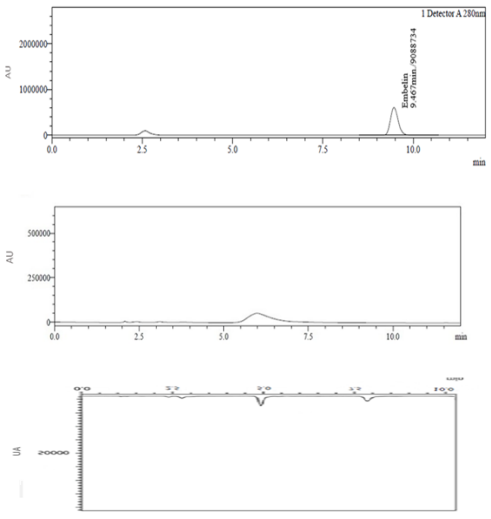

Figure 4. Representative HPLC chromatogram obtained under optimized condition: (A) EMB working standard, (B) blank sample, and (C) placebo.

Linearity

The linearity was assessed across an EMB concentration range of 125 to 750 µg/ml. The HPLC was used to analyze each working solution in order to determine the linearity. The peak area was plotted against EMB concentration, and the regression line provided the calibration curve's correlation coefficient indicating a a proportional relationship between peak area and the concentration (Table 7). Chromatogram of EMB at increasing concentration are shown in Figures (6−11). The standard curve exhibited an excellent linearity (R^2 = 0.999), confirming a proportional relationship between peak area and concentration. As shown in Figure 5, the calibration curve followed the linear regression equation y = 55569x + 774858, where y represents the peak area and x represents the EMB concentration (µg/ml).

Table 7. The area of the each working concentration solution of EMB, mean ± SD (n = 3)[a]

Concentration (µg/ml)[b]	Area 1	Area 2	Area 3	Mean[c]	Std	%RSD[c]
125	6588773	6684556	6684552	665260.33	5531.59	0.83
250	9818538	9828625	9818236	978846.00	6102.98	0.62
375	12028879	12145866	12156355	121103 3.33	7081.31	0.58
500	14314425	14119999	14225546	142199 0.00	9724.93	0.68
625	21703500	21605866	21608445	216392 7.00	5563.99	0.26
750	23868857	23756632	23856693	238272 7.33	6172.24	0.26

[a]Triple injections (n=3) of each working standard solution using validated RP-HPLC method. [b]Concentration range: 0–750 µg/ml ; peak areas from EMB. [c]Mean ± SD; %RSD = (SD/mean × 100). All %RSD < 0.7%.

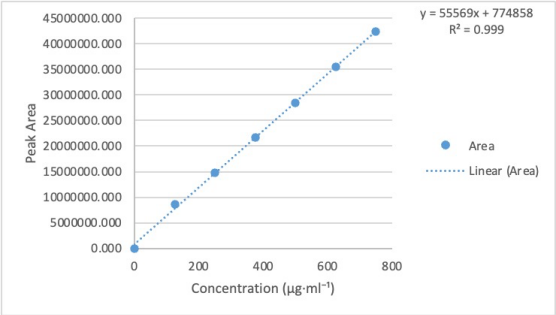

Figure 5. Calibration curve of EMB: peak area versus concentration of working standard solutions by RP-HPLC.

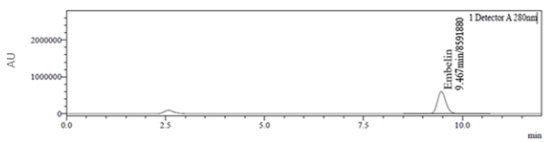

Figure 6. Representative HPLC chromatogram of EMB working standard (125 µg/ml) under optimized conditions (1 ml/min, 280 nm, 28 °C)

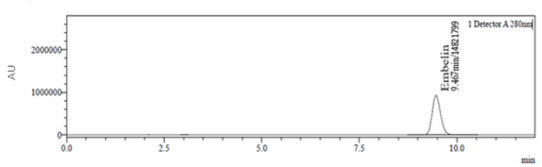

Figure 7 Representative HPLC chromatogram of EMB working standard (250 µg/ml) under optimized conditions

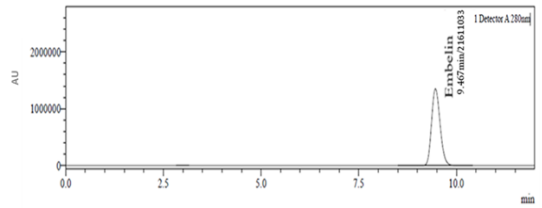

Figure 8. Representative HPLC chromatogram of EMB working standard (375 µg/ml) under optimized conditions

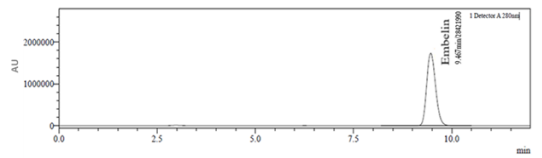

Figure 9. Representative HPLC chromatogram of EMB working standard (500 µg/ml) under optimized conditions

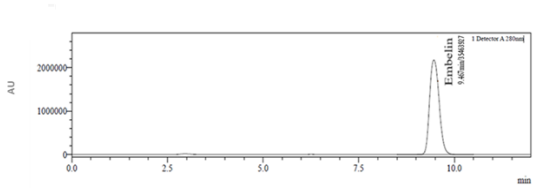

Figure 10. Representative HPLC chromatogram of working standard (625 µg/ml) under optimized conditions

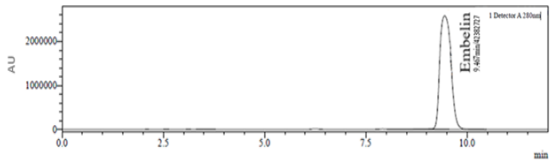

Figure 11. Representative HPLC chromatogram of EMB working standard (750 µg/ml) under optimized conditions

Limit of Quantitation and Limit of Detection

The sensitivity of the method was assessed by determining the LOQ and LOD. For the current method, the EMB LOD and LOQ were determined to be 0.06825 µg/ml and 0.20683 µg/ml, respectively (Table 8).

Table 8. LOD and LOQ parameters.

	Intercept
	596287
	598427
	598111
Average	597608
Std	1155.16
	Slope
	55855
	55844
	55853
Average	55850.7
Std	5.86
LOD	0.06825
LOQ	0.20683

Accuracy of the method

Recovery tests were performed using standard addition procedures to further evaluate the precision and reliability of the proposed method. Pre-analyzed samples were mixed with three different concentrations of pure EMB standard solution (75%,

100%, and 125%), and the total content was determined using developed method. methods. Each determination was repeated three times. The recovery results are shown in (supplementary Table S1) with EMB recoveries of 100.070 ± 0.139% at 75% level, 99.210 ± 0.058% at 100% level, and 100.190 ± 0.433 at 125% level was observed. The recovery results and %RSD showed that the proposed method had an acceptable level of accuracy for EMB. The % RSD values were below 2% at all levels, indicating the method's acceptable accuracy and precision.

Precision of the method

Intra-day precision (repeatability) was assess using three concentration levels: 75% (low, l375 µg/ml), 100% (middle, 500 µg/ml), and 125% (high, 625 µg/ml), each analysed six times. In terms of % RSD, the intra-day precision was less than 1% (supplementary Table S2). Inter-day precision (ruggedness) was evaluated by analyzing the same concentrations six times over six consecutive days, with % RSD values below 2%. The results for intra-day and inter-day precision are shown in (supplementary Table S3).

Ruggedness

Ruggedness was evaluated by having two separate analysts perform the experiment under identical conditions. No significant statistical differences observed between operators. The % RSD values (Table 9) were less than (< 2 %), indicating that the developed method has acceptable ruggedness.

Table 9[a]. % recovery of the EMB for the ruggedness parameter mean ± SD (*n* =6).

Analysis 1		
Concentration (µg/ml)	**Recovered Concentration (µg/ml)[b]**	**% recovery[c]**
500	498.386	99.677
500	498.311	99.662
500	496.158	99.232
500	498.148	99.630
500	497.755	99.551
500	498.499	99.700
	Mean	99.575
	STD	0.176
	%RSD	0.177
Analysis 2		
Con.(µg/ml)	**Recovered Concentration (µg/ml)**	**% recovery**
500	495.315	99.063
500	490.196	98.039
500	498.189	99.638
500	498.679	99.736
500	498.341	99.668
500	495.972	99.194
	Mean	99.223
	STD	0.642
	%RSD	0.647

[a]Six replicate determinations (n=6) per analyst using same RP-HPLC method; ruggedness factor = different analysts. [b]Nominal concentration 500 µg/ml working standard. [c]% Recovery = (recovered/nominal × 100); %RSD = (SD/mean × 100). Both %RSD <2.0%.

Robustness

The robustness of the proposed method was evaluated to assess its reliability under minor deliberate variation in chromatographic conditions. The organic phase ratio was varied at 85:15, 90:10, and 95:5, and the and the flow rate was adjusted at 0.9, 1 ml/min, and 1.1 ml/ min. Table 10 presents the retention time of EMB under varied organic phase and flow rate condition, while Table 11 shows % recovery of EMB for robustness. The % recovery remained within the acceptable range 98–102%, with %RSD less than 2%, indicating that minor variation in organic phase did not significantly affect the analytical performance of the method. Similarly, the % recovery remained within the acceptable range, and the %RSD was below 2%, demonstrating that method is accurate and robust under slight chromatographic fluctuations.

Table 10. Retention time of EMB for robustness parameters

Chromatographic Parameter	**Variation**	**Retention time**
Organic phase ratio	85:15	9.456
	90:10	9.464
	95:5	9.462
Flow rate (ml/min)	0.9 ml/min	9.482
	1 ml/min	9.461
	1.1 ml/min	9.463

Table 11. % recovery of EMB for robustness parameters mean ± SD (n =3)[a]

Change in Organic phase ratio[b]	**Recovered Concentration (µg/ml)**	**% recovery[c]**
85:15	496.15	99.23
	494.34	98.87
	498.38	99.68
	Mean	99.26
	STD	0.41
	%RSD	0.41
90:10	496.21	99.24
	498.13	99.63
	496.15	99.23
	Mean	99.37
	STD	0.23
	%RSD	0.23
95:5	498.050	99.61
	498.277	99.65
	496.407	99.28
	Mean	99.52
	STD	0.20
	%RSD	0.21
Change in flow rate	**Recovered Concentration (µg/mL)**	**% recovery**
0.9 ml/min	498.157	99.63
	498.870	99.77
	496.270	99.25
	Mean	99.55
	STD	0.27
	%RSD	0.27
1 ml/min	497.317	99.46
	498.870	99.77
	496.349	99.27
	Mean	99.50
	STD	0.25
	%RSD	0.26
1.1 ml/min	497.926	99.59
	495.217	99.04
	498.068	99.61
	Mean	99.41
	STD	0.32
	%RSD	0.32

[a]Triple determinations (n=3) using validated RP-HPLC method. [b]Change in organic phase ratio tested at 85:15; 90:10; and 95:5; flow rate robustness: 0.9-1.1 ml minl, [c]% Recovery = (recovered/nominal × 100); %RSD < 2.0%.

Application of the Developed Method for Validation of Lipid-Based Nano-formulations

The developed analytical method successfully determined the % EE of glycerosomes loaded with EMB drug. The entrapment efficiency of EGNs was found to be 98.36 ± 0.109% and their chromatogram is shown (Figure 12). It was demonstrated that the high entrapment of Embelin in glycerosmes is primarily due to presence of high glycerol and phospholipids, which enhance drug solubility and vesicle fluidity facilitating the formation of stable bilayer system. The chromatogram shows no significant change in retention time or analyte peak, leading to the conclusion that the devised approach was reliable and specific in determining the % entrapment efficiency.

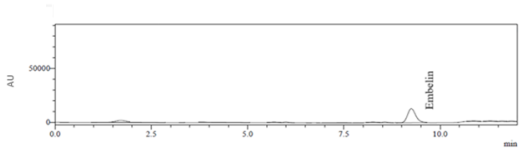

Figure 12. Representative HPLC chromatogram of Glycerosomes formulation containing EMB.

DISCUSSION

A hplc method is developed with the application of QbD for the optimization of chromatographic condition. The two dependent variables are used to see the responses of three independent variables. These are well optimized by using QbD approach. The optimized condition is reliable makes the method more precise and simple. The observed retention time mentioned above represent the affinity between mobile phase and stationary phase; that is the time taken by particular compound to pass the column. The observed retention time in this case was observed to be 9.467 min which is similar to the reference publication hence we can say that our compound is likely the same as the reference [22].

In our study we used 3 independent variables with 2 dependent variables as the optimization parameters, which was a handy and economical method for a rapid quantification of the compound present either in bulk or in the formulation. Hence, our method proved to be practical, rapid, accurate, time saving and economical as compared with the previously published papers. The HPLC method was validated according to ICH guidelines. The proposed HPLC method of EB analysis was linear, accurate, precise, robust and sensitive for the determination of EMB. The results of the validation parameters were in the acceptable range which indicates the HPLC method is robust and reliable for routine analysis of EMB in formulations. By the application of DoE, the time and effort that it usually takes in the selection of the exact combination of different variables to get a perfect solution to get and save time.

CONCLUSION

The method is an effective analytical technique for simultaneous estimation of EMB, in lipid-based nano-formulations. EMB was successfully determined in both bulk form and nano-formulation within ≤10 minutes, making the method more practical and economical. Furthermore, the developed method demonstrated good validation parameters that met all acceptance criteria recommended by ICH guidelines. Additionally, the method proved applicable by accurately estimating the amount of EMB entrapped in nano-formulation. Overall, the method has been shown to be reliable and rapid estimating drug content in different platforms, including combination nano-formulations as well as in vitro, and in vivo models in future work.

Conflicts of interest

No potential conflict of interest was reported by the author(s).

Authors contribution: All authors confirm their compliance with ICMJE authorship criteria. The contributions were distributed as follows: **Monika Dhaka**: study concept and design, experiment execution, data analysis and interpretation, manuscript preparation, and revision. **Md Habban Akhter**: study concept and design, data analysis and interpretation, manuscript preparation and revision. All authors have read and agreed to the published version of the manuscript.

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