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Development of a robust and reliable reverse-phase high-performance liquid chromatography (RP-HPLC) method using analytical quality by design principles for the accurate determination of esculin in its bulk form

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Abstract

Background Analytical quality by design is a proactive, holistic, and data-driven approach to quality that emphasizes risk assessment and management. This can lead to more robust and reliable methods than traditional approaches. Using principles of analytical quality by design for method development can help to assure the quality and consistency of analytical methods. This is important for the pharmaceutical industry, where accurate and reproducible analytical methods are essential for ensuring drug safety, shelf life, and efficacy. Esculin is a naturally occurring derivative of coumarin that is found in the stems of the plant Aesculus indica. The present study describes the use of an analytical quality by design approach to develop and validate a reliable RP-HPLC method for the analysis of esculin bulk form.

Result A central composite design was employed to optimize the percent of methanol in the mobile phase and flow rate for the analysis of a compound esculin using the RP-HPLC method. The optimized conditions were 43% methanol and 0.9 ml/min flow rate, with a retention time of 3.78 min, and Phenomenex Luna (5 µm × 250 mm, 4.6 mm) column was used. The method was found to be linear with a correlation coefficient of 0.9998 for a concentration range of 4–20 µg/ml. The parameters of the system suitability test were within the acceptable range (0.0612–0.1398%), and the precision for both intra-day and inter-day measurements was below 2%. The robustness and ruggedness of the method were also good, with changes in the flow rate and mobile phase composition having a minimal impact on the method's performance. The limit of detection (LOD) and limit of quantification (LOQ) values were reported to be 0.82891 µg/ml and 2.511 µg/ml, respectively. The validation parameters of the method adhered to the specified limit following the ICH guidelines.

Conclusion In summary, an AQbD-based efficient and robust RP-HPLC chromatographic method has been developed for the quantification of the esculin compound. The method is linear, precise, and reproducible, and it

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has good LOD and LOQ values. The method could be used for repetitive analysis of the compound in pharmaceutical formulations.

Keywords Analytical quality by design, HPLC, ICH, Esculin

Background

Herbs and herbal products are very much exploited for their medicinal properties for different diseased conditions. And since it is intended to be consumed by humans as a supplement or for treating diseases, it should have a high-quality standard. To assure this quality standard, phytochemical investigation and standardization are carried out on these herbs and herbal products. Further marker-based standardization is gaining popularity for depicting the quality and authenticity of herbal pharmaceuticals, which is achieved with the help of instruments like high-performance liquid chromatography, high-performance thin layer chromatography for quantitative estimation, and techniques like infrared and ultraviolet for qualitative estimation [1–3]. According to the ICH Q8 guideline, a QbD is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management" [4]. AQbD is an extension of QbD principles applied to analytical method development for achieving a robust and rugged method that is operable within the design space and to rule out the time to revalidate over its lifecycle [5]. AQbD involves identifying an analytical target profile, and critical quality parameters, which then undergo risk assessment, screening, optimization, and formation of method operable design space to understand the interaction of method variables followed by method validation [6, 7].

Coumarins consist of a wide variety of phenolic compounds having various biological activities such as anti-inflammatory, anti-oxidant, anti-mutagenic, and anti-cancer activity. And this is possible because of the change in the basic ring structure of coumarin [8, 9]. Esculin, also known as 6,7-dihydroxy-coumarin 6-glucoside, is a derivative of umbelliferone that contains an additional glycosidic component attached to the C6 position [10]. It is a naturally occurring derivative of coumarin found in the stems of the plant *Aesculus indica,* belonging to the family Sapindaceae; Hippocastanaceae. *A. indica* is generally known as Indian horse chestnut. It also contains other phytochemicals apart from esculin like quercetin, aescin, and beta-sitosterol which are present in leaves while astragalin, rutin, and esculin are present in the stem. The extract obtained from the seeds of A indicia can be used against human epidermoid carcinoma of the nasopharynx and P-388 lymphocytic leukemia [11]. Analytical methods, including spectrophotometry, HPLC, HPTLC, and UPLC-ESI-MS/MS methods [12–15], were reported for the quantification of esculin in bulk and in different plant extracts, as per the literature survey.

The present study is carried out to develop, optimize and validate the HPLC method for quantifying esculin in bulk by the QbD approach.

Methods

Chemicals

Esculin with a purity value of 98% was obtained from Research Lab Fine Chem Industries in Mumbai, India (see Fig. 1). Merck Mumbai Pvt. Ltd. supplied the HPLC grade of methanol. The Merck Direct Q Millipore water system was used to make ultra-pure water. We purchased 85% LR-grade orthophosphoric acid from S.D. Fine Chem Limited in Mumbai. The additional chemicals and reagents employed in the research were of high-quality analytical grade.

Instrument

The chromatographic process was designed utilizing a Shimadzu Prominence RP-HPLC system (Shimadzu, Japan) with Lab Solution version 1.25 software and with binary pumps (LC20AD.) with a degasser (DGU20A5.), PDA Detector (SPD-M20A.), auto-sampler (SIL 20AC HT.), and column oven (CTO-10AS).

Chromatographic condition

The chromatographic analysis was carried out by instilling 10µL of esculin solution into the HPLC system, which was attached to a C-18 analytical column Phenomenex Luna (5 µm × 250 mm, 4.6 mm), which was kept at an ambient temperature of 35 °C and operated at a flow rate of 1 ml/min initially. The mobile phase consisted of methanol and 0.1% orthophosphoric acid, which was employed to keep the pH constant.

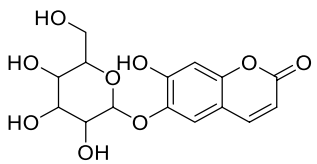

Fig. 1 Chemical structure of esculin

Sample preparation

A stock of standard esculin solution has been produced by dissolving precisely weighed 10 mg of esculin in 10 ml of (HPLC grade) methanol using a sonicator. The stock solution was then attenuated to a sub-stock concentration of 100 µg/ml. By adding methanol to 1 ml of sub-stock solution, a 10 µg/ml solution was obtained which was then passed through a 0.45 µm membrane filter before filling it in the vials.

Preparation of 0.1% orthophosphoric acid

Orthophosphoric acid (0.1 ml) was pipetted out in 100 ml of volumetric flask and volume was made up with Millipore water followed by sonication for 5 min for complete mixing.

Detection wavelength selection

Esculin solution of 10 µg/ml was scanned in the 200–400 nm range, and the maximum wavelength of 340 nm was chosen as the detection wavelength.

AQbD-based method development

Identifying analytical target profile and critical quality parameters

The analytical target profile (ATP) is a set of measures that describe what is to be assessed as well as the method's performance requirements. Critical quality parameters can also be defined as product qualities that should be maintained within a specified limit to attain a decent shelf life. Method properties and method parameters are included in CQP for analytical procedures. Organic modifier ratio in the Mobile phase, buffer pH, diluent, column type, elution method, flow rate, and column temperature are all included in the CQP for the HPLC process [16, 17].

Risk assessment

The applicable ICH recommendations (Q9 and Q10) include quality risk management and pharmaceutical quality system techniques. Risk assessment, as defined by the ICH Q9 guidelines, is a proficient method for analyzing, monitoring, and reviewing quality hazards all over the product life cycle. A risk analysis was performed to identify crucial attributes that could affect the analytical method and included in the design and was studied for its effects on the method's final performance [18].

Method optimization using the design of experiments

With the design of experiments (DoE), it is possible to formulate a mathematical relationship that relates the dependent variable to each of the independent variables, including the linear, quadratic, mean, 2FI, and interaction terms in the equation for regression analysis, by using the optimization design, which can produce better results with fewer trials [19–21]. In the current study, central composite design (CCD) in response surface methodology was used at two levels to optimize the analytical condition, which yielded 11 runs.

The setting of method operable design region (MODR)

The MODR, which is based on the output of DoE, is the optimal range of operation for the dependent variables that consistently yields the results that fulfill the objectives established in the analytical target profile [22] once the predicted 11 runs were performed on the instrument and the obtained data were analyzed using analysis of variance (TWO-WAY ANOVA), regression value, and 3-D response surface plot, MODR was established based on the requirements of critical quality parameters which yields multiple solutions of optimized chromatographic conditions.

Method validation

The RP-HPLC method underwent validation per ICH Q2 (R1) guidelines. System suitability, linearity, LOD, LOQ, precision, robustness, and ruggedness has been validated [23, 24].

Results

Previous research demonstrated the utilization of methanol and acetonitrile as organic modifiers, along with orthophosphoric acid, acetic acid, and other acids, to modify ph. In addition, optimal chromatographic parameters such as flow rate and oven temperature were noted, and preliminary trials were conducted using different mobile phase compositions based on these results. Furthermore, previously reported literature data reveals a graph with an unclear peak and a longer retention time, as well as the utilization of a higher flow rate of solvent. Therefore, the mobile phase composition of methanol as an organic modifier and 0.1% orthophosphoric acid was chosen, along with a suitable flow rate and column oven temperature.

Identifying analytical target profile and critical quality parameters

The selected analytical target profiles (ATPs) for optimizing the High-Performance Liquid Chromatography (HPLC) chromatographic conditions were the time it takes for a compound to elute (retention time) and the efficiency of the separation process (theoretical plates). And based on the determined analytical target profile, the critical quality parameters identified were the flow rate and the % organic modifier in the composition of the mobile phase.

Method optimization using the design of experiments

A central composite design consisting of 11 trials was employed to study the influence of two factors, the percent of organic modifier(methanol) in mobile phase composition (X1) and flow rates (X2) which are independent variables, on two response variables, retention time (R1) and theoretical plates(R2) as dependant variables. The responses of these 11 trials are summarized in Table 1. The data were analyzed using the TWO-WAY ANOVA test for the quadratic model, and mathematical expressions were derived to understand the correlation between the independent and dependent variables which is depicted in Table 2. Response surface plots were generated to visualize the effects of each factor on the responses as shown in Figs. 2 and 3 for retention time and theoretical plates, respectively.

The analysis of the plots revealed that decreasing the amount of variable X1 (concentration of methanol in the 0.1% orthophosphoric acid buffer) led to an increase in responses R1 and a decrease in responses R2 up to a level, suggesting an increase in retention time and a reduction in theoretical plates, whereas decreasing the intensity of variable X2 increases the value of both the responses R1 and R2. The polynomial equation (for coded values) for retention time equals $= 2.95 - 0.6865 \times A - 0.8717 \times B - 0.1107 \times AB + 1.17 \times A^2 + 0.1222 \times B^2$ and for theoretical plates is

$= 2377.61 + 614.13 \times A - 849 \times B - 320.20 \times AB + 1297.40 \times A^2 + 129.32 \times B^2$, where A (% methanol in mobile phase) and B (flow rate) are independent variables while AB, A^2, and B^2 are interaction and quadratic terms. A positive sign of the coefficient shows that the independent variable and the dependent variable have a synergistic effect. This means that when the independent variable lifts, the dependent variable also raises. A negative sign of the coefficient indicates that the independent variable and the dependent variable have an antagonistic effect. This means that when the independent variable increases, the dependent variable decreases. The larger the coefficient, the stronger the outcome of the independent variable on the dependent variable.

Table 1 Predicted combination of factors as per central composite design

Run	X1 (% of Methanol)	X2 (Flow rate)	R1 (Retention time)	R2 (Theoretical plates)
1	35	0.8	5.668	3700.34
2	35	1.2	4.11	2621.63
3	47.5	1.2	2.25	1674.26
4	47.5	1	2.9	2371.76
5	60	0.8	4.58	5632.22
6	60	1	3.38	4221.12
7	60	1.2	2.579	3272.73
8	47.5	1	3	2369.79
9	47.5	1	2.93	2400.86
10	35	1	4.88	3119.34
11	47.5	0.8	3.921	3330.04

The setting of method operable design region (MODR)

MODR is a multidimensional space that defines the range of acceptable operating conditions for a given method. It is created by combining and interacting with the independent factors that affect the method. The MODR can be used to select operating conditions that will ensure the desired quality of the results. The operating conditions for the HPLC method were chosen based on criteria that would fulfill the desired analytical target profile and critical quality parameter. The Method Operable Design Region (MODR) was represented by an overlay plot in Fig. 4, with the yellow region indicating the optimized zone. This MODR region was set based on the ranges applied to the constraints (independent variable) to achieve a specific goal as given in Table 3. The predicted solution was represented by a flag in this zone which showed a good desirability value of 0.924 predicted by design expert software.

To evaluate the predicted solution, the given set of conditions was implemented on the HPLC instrument. And based on the relative standard deviation values, the predicted solution was deemed as an optimized HPLC method. Criteria for optimization were good reproducibility of the peak area, retention time, theoretical plates and tailing factor which were set based on the

Table 2 Two-way ANOVA for Quadratic model

Response	P value	Model significance	Polynomial equation
R1	0.0209	Significant	$= 2.95 - 0.6865 \times A - 0.8717 \times B - 0.1107 \times AB + 1.17 \times A^2 + 0.1222 \times B^2$
R2	0.0001	Significant	$= 2377.61 + 614.13 \times A - 849 \times B - 320.20 \times AB + 1297.40 \times A^2 + 129.32 \times B^2$

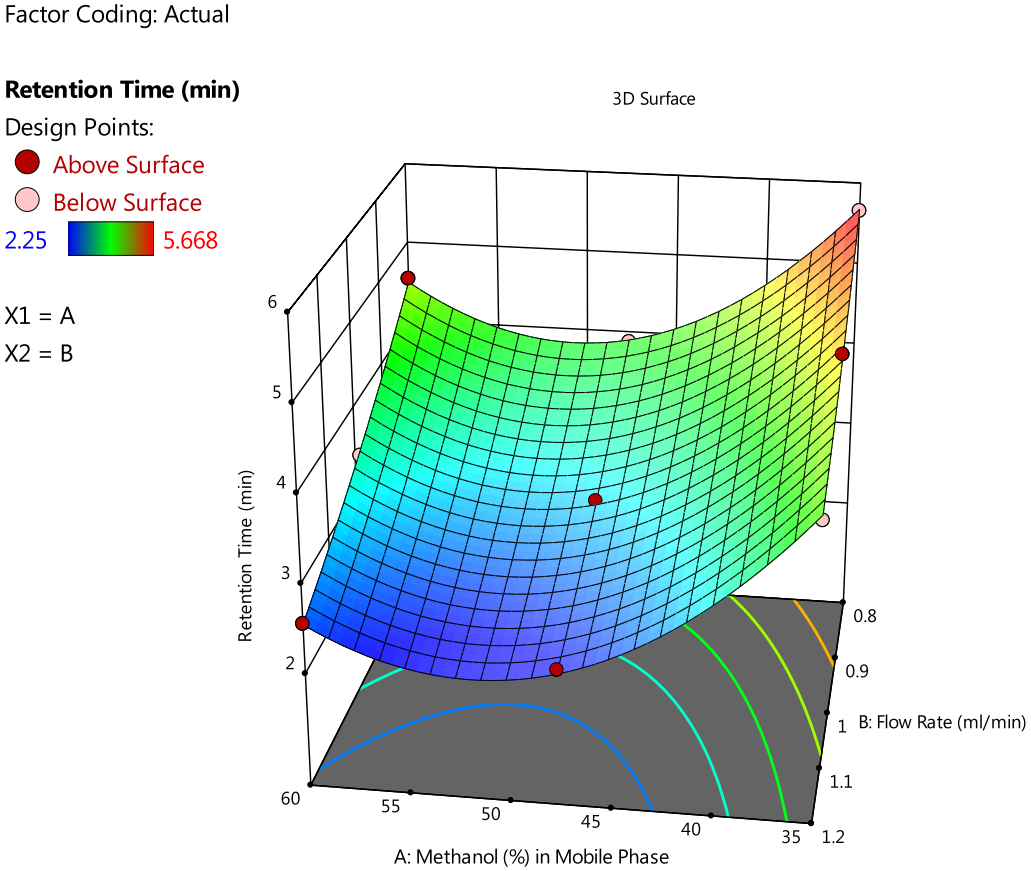

Fig. 2 Response surface graph for studying the combined impact of independent variables on dependent variable retention time

preliminary data obtained during method development and literature review. Details are provided in Table 4. The chromatography of the optimized run is shown in Fig. 5. Further validation was conducted using the set chromatographic conditions given in Table 5.

Method validation

The RP-HPLC technique that was developed underwent validation to showcase its fitness for the proposed purpose, as outlined in the ICH Q2 (R1) guidelines. The validation factors of the proposed RP-HPLC method are presented in Table 6, and it can be observed that these parameters confine the prescribed standard limits mentioned in the ICH Guidelines.

The developed method was confirmed to be suitable for its intended purpose by calculating the Relative Standard Deviation (RSD) as a percentage for various constraints such as peak area, retention time (Rt), and tailing factor. The RSD values for peak area, retention time, and tailing factor were all found to be within acceptable limits, which were less than 2%. This means that the method is precise and reproducible.

A linear calibration curve was established for esculin using a specific concentration range (Fig. 6). The Limits of Detection (LOD) and Limits of Quantification (LOQ) were determined based on the linear regression analysis of the calibration curve. The LOD was found to be 0.82891 µg/mL and the LOQ was found to be 2.511 µg/ml. This means that the method is sensitive enough to detect esculin at low concentrations and can be used to quantify esculin at higher concentrations.

The precision of the analytical method was assessed through reproducibility and repeatability. Reproducibility is the ability to obtain similar results when the method is executed multiple times under identical conditions, while Repeatability is the ability to obtain matching results

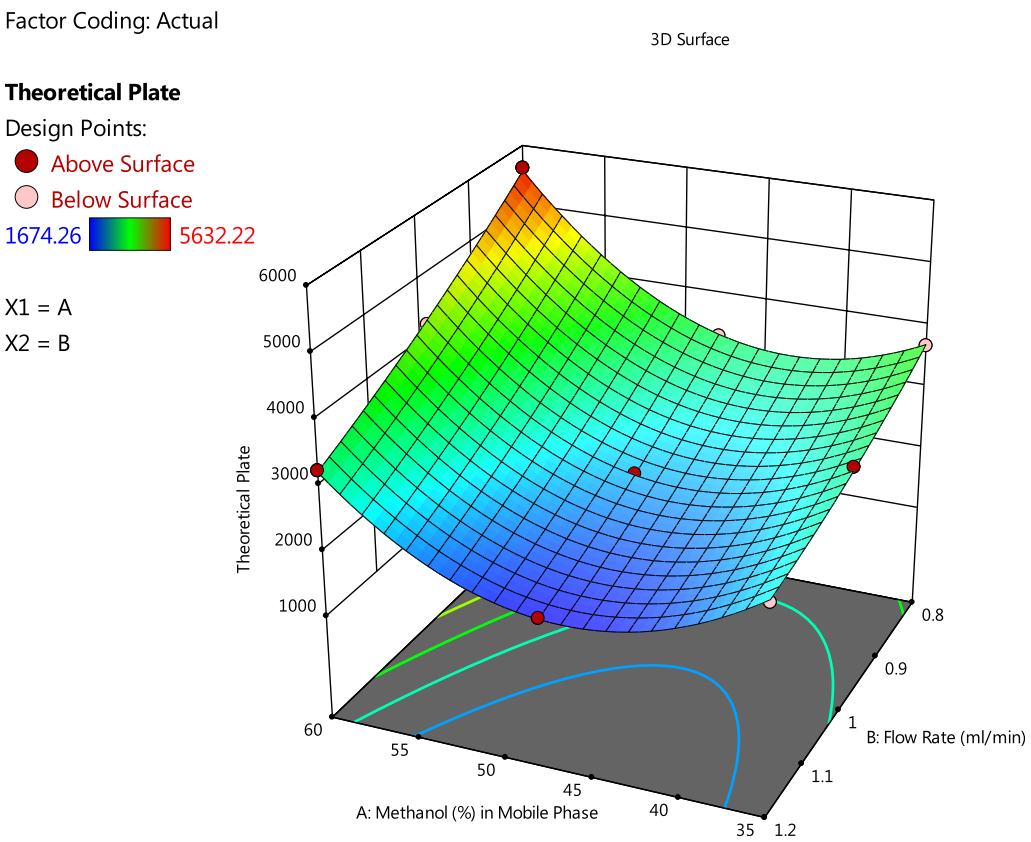

Fig. 3 Response surface graph for studying the combined impact of independent variables on dependent variable theoretical plates

when the method is implemented multiple times by different people under different conditions. The intra-day and inter-day RSD values for esculin were both low, indicating that the method is reproducible and repeatable. The method was robust and rugged, with % RSD values of 0.90% and 1.10% which are less than the given limit.

Overall, the results of the system suitability tests indicate that the developed RP-HPLC AQbD-based method is suitable for the analysis of esculin.

Discussion

Analytical Quality by Design (AQbD) principles were used to develop an Analytical Target Profile (ATP) for the quantification of esculin using HPLC. The ATP focused on retention time, theoretical plates, and peak asymmetry. The critical quality parameters (CQPs) that affect the ATP were identified as the flow rate and % of methanol in the mobile phase.

A central composite design was used to study the effect of the CQPs on the ATP. Statistical analyses, such as TWO-WAY ANOVA, were performed to thoroughly examine the CQPs. Polynomial equations and response surface plots were created to understand the correlation between analytical parameters and critical quality parameters.

The optimized chromatographic settings were determined using the MODR (Movable Origin of Desirability) method. The optimized conditions were then used to analyze esculin in bulk. The retention time for esculin was found to be 3.78 min. The technique showed linearity in the constraint of 4–20 µg/ml with a correlation coefficient of 0.9998. The method demonstrated good precision with %RSD values for repeatability, intra-day, and inter-day precision all less than 2%. The Limits of Detection (LOD) and Limits of Quantification (LOQ) were determined to be 0.82891 µg/ml and 2.511 µg/ml, respectively. The method development process adhered to the ICH guidelines.

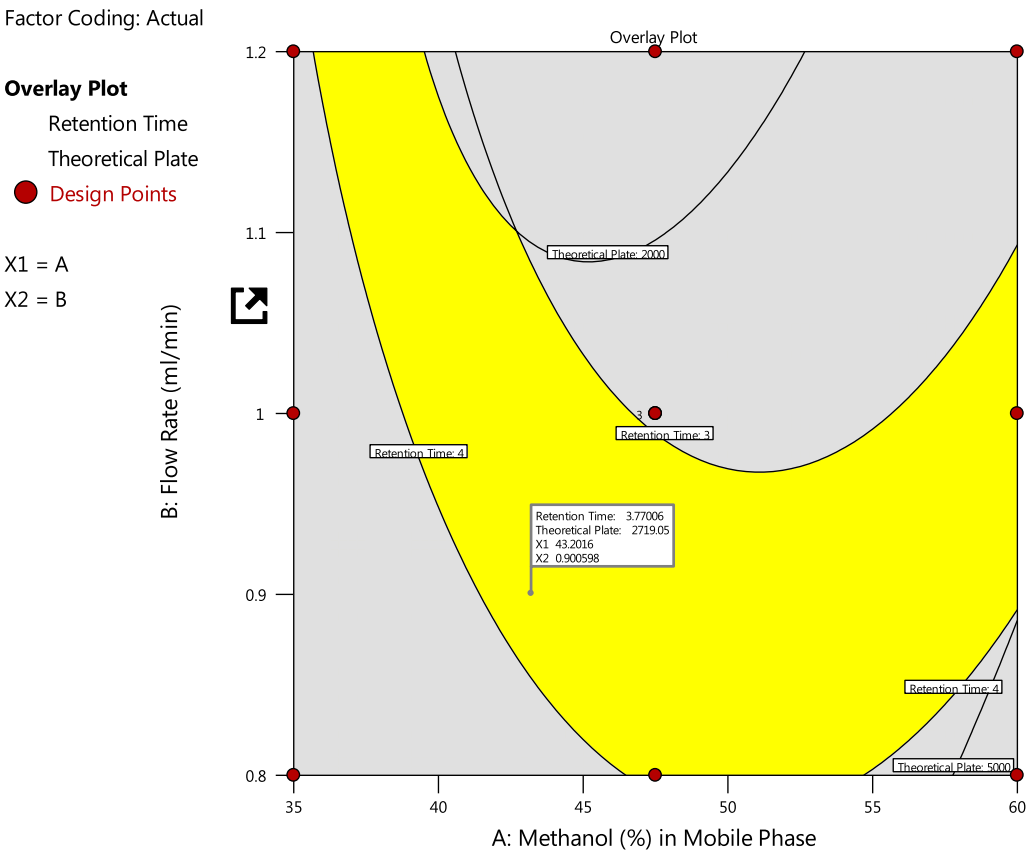

Fig. 4 Overlay plot depicting MODR for esculin

Table 3 Set ranges of MODR region

Name	Goal	Lower limit	Upper limit	Importance
A: Methanol (%) in mobile phase	Maximize	40	50	3
B: Flow rate	Is in range	0.8	1.2	3

Table 4 Optimized run

Parameter	Observations		
	Predicted values	Observed values	% RSD
Methanol (%) in mobile phase	43.2	43	0.328886875
Flow rate (ml/min)	0.901	0.9	0.07856742
Retention time (min)	3.77	3.78	0.187065286
Theoretical plates	2719.02	2727.43	0.218035588

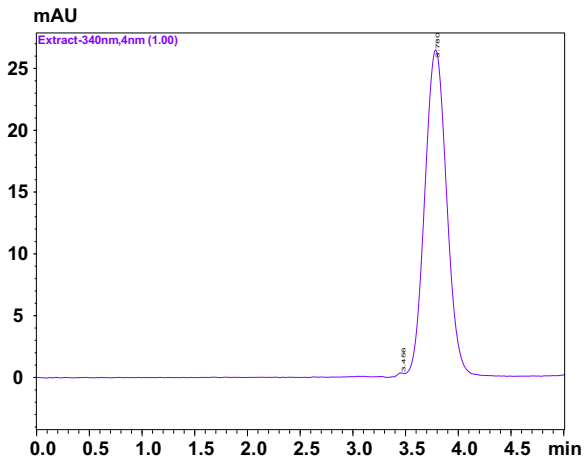

Fig. 5 Chromatograph of the optimized run

Table 5 Optimized chromatographic conditions

Parameters	Conditions
Flow rate	0.9 ml/min
Temperature	35 °C
Ratio	43:57 (Methanol:0.1% OPA)
Injection volume	10 µl
Retention time (%RSD, $n=6$)	3.78 min
Area (%RSD, $n=6$)	209,221
Tailing factor	1.336

Table 6 Summary of validation parameters

Validation parameters	Results
Linearity	
Correlation coefficient	0.9998
Range	4–20 µg/ml
Sensitivity	
LOD (µg/ml)	0.82891
LOQ (µg/ml)	2.511
Precision	
Inter-day (%RSD)	0.665
Intra-day (%RSD)	0.612
Robustness	0.9089
Flow rate (%RSD)	0.7385
Ratio (%RSD)	0.9365
Temperature (%RSD)	1.0516
Ruggedness	
Change in analyst (%RSD)	1.1011
System suitability	1.24
Retention time (%RSD)	0.1398
Peak area (%RSD)	0.7146
Theoretical plates (%RSD)	0.0612

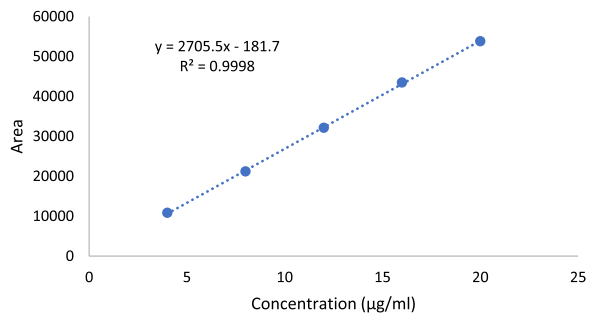

Fig. 6 Calibration curve of esculin

Conclusion

Overall, the results of this study showed that the DoE was an effective way to optimize the conditions for an RP-HPLC method for the analysis of a pharmaceutical compound. The developed method was found to be linear, accurate, precise, reproducible, robust, and rugged, and there is still scope to test the method for routine analysis which was limited in this study due to the non-availability of the esculin in commercial dosage form.

Abbreviations

RP-HPLC	Reverse-phase high-performance liquid chromatography
AQbD	Analytical quality by design
LOD	Limit of detection
LOQ	Limit of quantification
QbD	Quality by design
ATP	Analytical target profile
CQP	Critical quality parameters
HPTLC	High-performance thin layer chromatography
UPLC-ESI-MS/MS	Ultra performance liquid chromatography-electron spray ionization mass spectrometry/mass spectrometry
HPLC	High-performance liquid chromatography
CCD	Central composite design
MODR	Method operable design region
DoE	Design of experiments
TWO-WAY ANOVA	Analysis of variance
RSD	Relative standard deviation

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Author contributions

We have assured that "all authors have read and approved the manuscript." All the authors have equal contribution and participation in this research work. SP has reviewed all manuscripts on "Development of a robust and reliable reverse-phase high-performance liquid chromatography (RP-HPLC) method using Analytical Quality by Design principles for the accurate determination of esculin in its bulk form"; he had completed his work under the supervision of Dr. AA and Dr. PS. RG also helped him in their research work and guides to resolve the complications.

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Availability of data and materials

The research work has been carried out by us, and we assure you that it can be provided to you whenever required.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors declare no conflict of interest.

Competing interests
No competing interests to declare.

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