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STRATEGIC OPTIMIZATION OF HPTLC METHOD FOR BERBERINE QUANTIFICATION IN B-CYCLODEXTRIN INCLUSION COMPLEX USING DESIGN OF EXPERT

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ABSTRACT
Wound healing involves complex stages like hemostasis and inflammation. Berberine, a natural compound, exhibits antimicrobial and anti-inflammatory properties, aiding healing. Nanotechnology enhances the delivery of berberine, highlighting the need for a reliable analytical method for its quantification. In this study, we developed and validated a High-Performance Thin-Layer Chromatography (HPTLC) method for the simultaneous quantification of berberine in β-cyclodextrin inclusion complex nanoparticles. The method development employed a Box-Behnken Design (BBD) to optimize chromatographic conditions, focusing on the volume of methanol, saturation time, and migration distance. The optimized HPTLC conditions utilized a mobile phase of ethyl acetate, methanol, and water (7:2:1 v/v), achieving a precise separation of berberine with an Rf value of 0.52 ± 0.01. Method validation, following ICH guidelines, confirmed linearity (200-1000 ng/band, R^2 = 0.999), precision (intra-day and inter-day %RSD < 2%), accuracy (recoveries between 97.1% and 98.5%), robustness, and solution stability (%RSD < 2% after 48 hours). This validated method effectively quantified berberine in nanoformulations, highlighting its potential as a robust analytical tool for evaluating berberine-based wound healing therapies.
Keywords: Berberine, B-Cyclodextrin Inclusion Complex, Design of Expert, Box-Behnken Design, Analytical Method Validation.
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INTRODUCTION

Wound healing is the intricate process by which the body repairs damaged tissues. It involves several stages: hemostasis, inflammation, proliferation, and remodeling. Blood clotting, immune response, and tissue regeneration mechanisms play vital roles.[1] Factors like nutrition, age, and underlying health conditions influence the speed and effectiveness of healing. Conventional wound healing treatments, like antibiotics and antiseptics, may lead to antibiotic resistance and allergic reactions.[2] They can delay wound closure by inhibiting natural healing processes and may cause tissue damage. Additionally, frequent dressing changes can disrupt the healing environment, leading to increased pain and risk of infection.[3] Hence, there is a growing need for alternative therapeutic strategies that are safe, effective, and convenient. Berberine (Fig.-1), a compound found in several plants like goldenseal and barberry, shows promise in wound healing due to its antimicrobial, anti-inflammatory, and antioxidant properties. Research suggests berberine promotes collagen production and accelerates wound closure.[4,5,6]

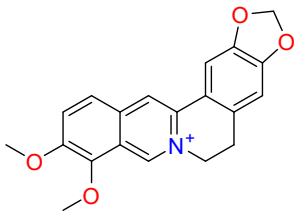

Fig.-1: Chemical Structure of Berberine

Nanotechnology's application in wound healing encompasses the utilization of nanoparticles to administer medications, foster tissue regeneration, and fabricate sophisticated dressings aimed at expediting and

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enhancing the efficacy of wound recovery.[7, 8] Nanoparticles represent a promising avenue in wound healing by encapsulating berberine, a natural compound renowned for its therapeutic properties. These nanoparticles serve to augment berberine's stability, solubility, and bioavailability, thereby facilitating its controlled release precisely at the wound site.[9] Consequently, endeavors are underway to devise adept delivery systems incorporating this berberine formulation within our research facilities, with the objective of validating their efficacy in wound healing. However, prior to embarking on exploratory investigations into delivery systems, the establishment of a suitable analytical method is imperative. This method plays a pivotal role in accurately separating and quantifying berberine within the prepared nanoformulation, thus laying the groundwork for comprehensive assessment and refinement of the proposed wound healing strategy. A range of analytical techniques, including high-performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), and high-performance thin-layer chromatography (HPTLC), can be utilized for the quantification of berberine, either alone or in combination with other substances. In a study by Garhwal S. *et al.,* the analysis of berberine content in the roots and bark of three Himalayan *berberis* species was conducted using the HPTLC method. Chromatography was carried out on glass-backed silica gel 60 F_{254} plates with a solvent system consisting of n-propanol: water: formic acid in a ratio of 90:8.0:0.4. The R_f value was determined to be 0.29.[10] In a study conducted by Satija S. et al., berberine was quantified in *Tinospora cordifolia* plants. The chromatographic separation was carried out on HPTLC plates coated with silica gel 60 F_{254}, using a mobile phase consisting of methanol, acetic acid, and water in an 8:1:1 (v/v) ratio. Detection was performed densitometrically at 366 nm, with the Rf value for berberine recorded at 0.71 ± 0.02.[11] Satija, S. *et al.* carried out the simultaneous quantification of berberine and rutin in both medicinal plants and pharmaceutical preparations. The chromatographic separation employed a mobile phase made up of n-hexane, ethyl acetate, glacial acetic acid, and methanol in a 10:1.1:1.1:2.5 (v/v) ratio. Densitometric analysis was performed at 254 nm, with the Rf values for berberine and rutin being 0.67 ± 0.02 and 0.47 ± 0.02, respectively.[12] In another study by Shailajan, S. *et al.,* authors reported quantification of the pharmacologically active compounds quercetin and berberine was performed utilizing High-Performance Thin-Layer Chromatography (HPTLC) from a multi-herbal formulation, Pushyanuga Churna. Chromatographic separation was achieved on silica gel 60F_{254} precoated HPTLC plates with toluene: ethyl acetate: methanol: formic acid (in a ratio of 6:6:2:1, v/v/v/v) as the mobile phase. The refined method achieved separation of the biomarkers, with Rf values of 0.24 for berberine and 0.61 for quercetin, respectively.[13] However, these reported methods have utilized complex solvent mixtures as the mobile phase, and none of the studies have reported the estimation of berberine in nanoformulations such as β-cyclodextrin inclusion complexes. Moreover, the reported methods lack an optimization process using the Quality by Design approach. On the other hand, both HPTLC (High-Performance Thin-Layer Chromatography) and HPLC (High-Performance Liquid Chromatography) play crucial roles in quantitative analysis. While HPLC is widely used, HPTLC offers distinct advantages. Its rapid analysis, cost-effectiveness, and ability for high-resolution, sensitive measurements make it particularly attractive. HPTLC enables simultaneous analysis of multiple compounds, a capability not as easily achieved with HPLC. Furthermore, it requires minimal sample volume, thus reducing material consumption and costs. With its precise quantification capabilities, HPTLC emerges as a favorable choice for researchers seeking efficient, economical, and accurate analytical methods. Consequently, the current research endeavors aim to develop and validate a suitable HPTLC analytical method for berberine, while concurrently quantifying this compound in cyclodextrin inclusion complexes.

EXPERIMENTAL

Chemicals and Reagents

Berberine and β-cyclodextrin (Sulphobutyl ether) were procured from Sigma Aldrich, Mumbai, India. Analytical-grade ethyl acetate, methanol, and acetonitrile were purchased from M/s Merck Ltd., Mumbai, India. All other solvents or reagents employed were of either pharmaceutical or analytical grade.

HPTLC Method Development

A method employing High-Performance Thin-Layer Chromatography (HPTLC) was developed to accurately quantify berberine. The HPTLC system, equipped with a Linomat 5 sample applicator from Camag, Switzerland, facilitated precise application of standard and sample solutions using a Hamilton

syringe (100 mL) with slit dimensions of 5.00 mm × 0.45 mm. Probing of samples occurred at a speed of 20 mm/s, with a data distinction of 100 µm/step, at a wavelength of 254 nm, managed by CATS version 3 software (Camag, Switzerland). Chromatographic separation of the bioactive compound utilized a pre-coated silica gel aluminum plate 60 F254 measuring 20×10 cm, with a thickness of 1 mm. TLC development was conducted in a flat-bottomed, twin-trough TLC developing chamber from Camag, Muttenz, Switzerland, provided by Anchrom Enterprises (I) Pvt. Ltd. in Mumbai, India. Samples were applied 10 mm from the bottom and side edges, with a 10 mm distance between tracks, forming bands. Each band was 7 mm long on the pre-coated TLC plate.[14]

Preparation of Standard Solution

A stock solution of berberine was generated by careful measurement of 10 mg of the bioactive compound into a 10 mL volumetric flask. Methanol was added to the flask, up to the mark, resulting in a final concentration of 1000 µg/mL. From this stock solution, a working standard solution with a concentration of 100 µg/mL was achieved through dilution with methanol. Sequential concentrations of the stock solution were then performed to generate various working standards with concentrations ranging from 200 to 1000 ng/band. Before chromatographic analysis, all samples underwent filtration using a 0.22 µm syringe filter.[15]

Selection of Appropriate Mobile Phase

Experimental trials were initiated to evaluate the bioactive compound on a TLC plate coated with 60 F254, employing different mobile phases. The choice of solvent systems was guided by factors such as polarity, dielectric constant, and insights from relevant literature. The mobile phase components were combined within a development chamber and pre-saturated for 8 minutes prior to application. Each individual run utilized approximately 30 µL of the mobile phase. Subsequently, the plates were dried in a hot air oven at 30°C and analyzed using an HPTLC method. The optimized mobile phase consisted of a blend of ethyl acetate, methanol, and water in a ratio of 7:2:1 (v/v). However, to comprehensively understand the combined impact of various HPTLC chromatographic factors on the response, a multivariate approach employing the Design of Experiments (DoE) concept was employed.

Software-Aided Method Optimization

To assess the primary and quadratic effects of factors on the retention factor (Rf) of berberine, we utilized a specialized design known as the Box-Behnken Design (BBD) within DoE software version 13.0 for the optimization procedure.[16-18] The Box-Behnken design, unlike the Central Composite Design (CCD), efficiently explores response surfaces with fewer experimental runs, making it optimal for minimizing resources while maximizing information retrieval, ensuring robust process optimization. Initial experiments revealed that alterations in chromatographic conditions, notably variations in the volume of methanol within the mobile phase, migration distance, and saturation duration of the chromatographic chamber, noticeably affected the Rf value of berberine. Hence, these variables were identified as crucial factors influencing the Rf value of berberine. The optimization of the HPTLC method considered parameters such as the mobile phase composition, represented by the volume of methanol (X1), chromatographic chamber saturation time (X2), and migration distance (X3). The responses analyzed were the Rf values of berberine, detailed in Table-1. For optimization purposes, the methanol volume (X1) was varied within the range of 1.5, 2, and 2.5 mL. Similarly, the chamber saturation time (X2) was adjusted to 10, 20, and 30 minutes, while the migration distance (X3) was set at 50, 70, and 90 mm, respectively. After obtaining the result, a polynomial expression was derived, and a comprehensive analysis of variance (ANOVA) scanning was conducted to identify the optimal levels of X1, X2, and X3 required to attain the anticipated outcome. The designated model was meticulously evaluated, focusing on the response associated with the Rf values of berberine.

Table-1: The Box-Behnken Experimental Design Incorporated Various Chromatographic Parameters for the Development of the Proposed HPTLC Method

Factor	Lower level (-1)	intermediate level (0)	Higher level (+1)
Volume of methanol (X_1)	1.5 mL	2 mL	2.5 mL
Saturation time (X_2)	10 min	20 min	30 min
Migration distance (X_3)	50 mm	70 mm	90 mm
Responses	R_f values of Berberine (Y_1)		

Preparation and Characterization of B-Cyclodextrin Inclusion Complex Nanoparticles

The inclusion complex comprising berberine and β-cyclodextrin was synthesized at a molar ratio of 1:1. Initially, a solution of β-Cyclodextrin in water with a concentration of 2 mM was prepared. Subsequently, 10 mg of berberine dissolved in 1 mL of absolute ethanol was added dropwise into the aforementioned β-Cyclodextrin solution while stirring continuously. The stirring was maintained at 400 rpm for a duration of 3 hours, and conducted in darkness to facilitate the inclusion reaction. Following the completion of the inclusion reaction, nanoparticles were formed utilizing the nanoprecipitation technique. Absolute ethanol (40 mL), acting as the non-solvent, was gradually added drop by drop into the solution containing the berberine/β-cyclodextrin inclusion complex under magnetic stirring at room temperature. The stirring was sustained at 400 rpm for an additional 10 minutes after the complete addition of ethanol. Consequently, nanoparticles spontaneously precipitated, yielding a suspension. The suspension was subjected to centrifugation to separate the berberine inclusion complex nanoparticles, followed by freeze-drying to obtain a dry powder. The resulting berberine/β-cyclodextrin inclusion complex nanoparticles (CINPs) were then stored at 4 ℃ for subsequent analysis. Physicochemical evaluation of the nanoparticles encompassed measurement of particle size, entrapment efficiency, zeta potential, and polydispersity index. These analyses were conducted to ascertain the characteristics and quality of the synthesized nanoparticles.[19, 20]

Sample Processing for Berberine/β-Cyclodextrin Inclusion Complex Nanoparticles

10 mg of dry powdered berberine/β-cyclodextrin inclusion complex nanoparticles were solubilized in 10 mL of methanol, resulting in a concentration of 1000 µg/mL. The solution was then subjected to sonication for a duration of 15 minutes to ensure uniform dispersion. Following sonication, the solution was diluted with methanol to achieve a concentration of 500 ng/band. Subsequently, the diluted solution underwent analysis to determine the berberine content within the prepared nanoparticles utilizing the proposed HPTLC method.[21]

Method Validation

Conforming to the guidelines set by the International Council for Harmonisation (ICH), the validation of the High-Performance Thin-layer Chromatography (HPTLC) method ensures the reliability and accuracy of analytical results. This comprehensive process entails assessing various parameters including linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy, robustness, and solution stability. By rigorously validating the HPTLC method, confidence is established in its suitability for the intended analytical purpose. This instills trust in the precision and consistency of the data generated through HPTLC analysis, affirming its utility as a dependable analytical tool.[22-26]

Linearity

In order to assess the linearity of the developed method, berberine stock solutions were prepared across a range of concentrations from 200 ng/band to 1000 ng/band, employing the mobile phase. Each concentration was subjected to six replicate determinations (n = 6) to ensure statistical robustness. Subsequently, the resultant peak areas were mapped relative to their respective test concentrations, and statistical interpretation was performed utilizing the least squares method. The evaluation of linearity encompassed the determination of key parameters including the regression coefficient (R^2) and the y-intercept of the linear regression plot, which elucidates the relationship between the response and concentration.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The analysis of limits of detection (LOD) and quantification (LOQ) for the HPTLC technique included utilizing the standard deviation method. Equations (1) and (2) were applied for the computation of these limits.

$$LOD = 3.3 \times SD/S$$
$$LOQ = 10 \times SD/S$$

Precision

To evaluate the precision of the developed method, three distinct concentrations of berberine equating to 400, 600, and 800 ng per band were assessed at various intervals throughout the day to determine intra-day

consistency. The analysis was repeated the next day to assess consistency between days. To validate precision, both the standard deviation (SD) and the percentage relative standard deviation (% RSD) were computed. These statistical metrics provide insight into the consistency and reproducibility of the analytical results, ensuring the reliability of the method across different experimental conditions and time periods.

Accuracy

To assess accuracy, the proposed method was employed to analyze placebos containing predetermined amounts of berberine across the method's linear interval. These amounts corresponded to 50, 100, and 150% of the nominal levels. The percentage recovery (%), which indicates accuracy, was computed for both standard lopinavir and prepared nanoparticles.

Robustness

The study investigated the influence of fine-tuning method parameters, including modifications to the mobile phase volume, saturation time, and migration distance. To gauge the impact of these adjustments on the R_f values and peak areas, the percentage relative standard deviation (% RSD) was calculated for each parameter.

Solution Stability

The stability of berberine standard solutions was verified by assessing their concentrations (500 ng/band) after storage for 48 hours at 4°C. Utilizing the proposed method, the analysis demonstrated % RSD values below 2%, affirming the stability of berberine solutions during the designated period under refrigerated conditions.

RESULTS AND DISCUSSION

Software-Aided Method Optimization

The optimization process employed the Box-Behnken design methodology, allowing for a systematic exploration of design space. Three independent variables—methanol volume, saturation time, and migration distance—were each varied across three levels: low, medium, and high, denoted as -1, 0, and +1, respectively. These variables were selected based on their presumed influence on the response variable, which in this case was the R_f values of berberine (Y1). To comprehensively understand the interplay between these variables and their impact on the response, a Design of Experiments (DoE) programmer was utilized. This tool facilitated the identification of critical values necessary to achieve the desired outcomes, providing valuable insights into the experimental parameters. The R_f values of berberine (Y1) resulting from each chromatographic trial were recorded and organized, with the findings succinctly summarized in Table-2.

Table-2: The Box-Behnken Experimental Design Was Implemented to Optimize the Parameters For the Proposed Hptlc Method, Yielding Specific Outcomes

	Factor 1	Factor 2	Factor 3	Response 1
Run	A: Volume of methanol	B: Saturation time	C: Migration distance	RF (Berberine)
	mL	min	mm	
1	2	10	50	0.5
2	2.5	20	50	0.69
3	2	20	70	0.52
4	1.5	30	70	0.49
5	1.5	10	70	0.43
6	2.5	30	70	0.73
7	2	30	90	0.61
8	2.5	20	90	0.71
9	2.5	10	70	0.66
10	2	20	70	0.53
11	2	30	50	0.57
12	2	20	70	0.52
13	1.5	20	50	0.46
14	2	10	90	0.51
15	1.5	20	90	0.48

The validation of the model was conducted through analysis of variance (ANOVA) using Design Expert software version 13.0, which facilitated the development of the experimental matrix for the response surface methodology. In ANOVA analysis, the desirability of a significant model is paramount. The statistical details of the applied design are succinctly presented in Table-3. Furthermore, the agreement between the Predicted R^2 of 0.9710 and the Adjusted R^2 of 0.9937 is noteworthy, with the disparity being less than 0.2. This consistency underscores the significance of the model in the separation process. Moreover, the coefficient of variation (% CV), serving as an indicator of model reproducibility, is ideally maintained below 10%. In our investigation, the % CV stands at 1.36 for response Y1, well within the acceptable range. To assess the lack of fit of the model, the pure error of the experiments can be evaluated using center points. In our current study, this lack of fit was thoroughly examined, revealing a P value of < 0.0001 for the response. This outcome indicates an adequate and well-fitting model ($P < 0.05$) without any discernible lack of fit.

Table-3: Overview of the Analysis of Variance (ANOVA) Results for the Box–Behnken Experimental Design Concerning the Proposed HPTLC Method, (Y1= Rf Berberine)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1290	9	0.0143	245.72	< 0.0001	significant
A-Volume of methanol	0.1081	1	0.1081	1853.36	< 0.0001	
B-Saturation time	0.0112	1	0.0112	192.86	< 0.0001	
C-Migration distance	0.0010	1	0.0010	17.36	0.0088	
AB	0.0000	1	0.0000	0.4286	0.5416	
AC	0.0000	1	0.0000	0.0000	1.0000	
BC	0.0002	1	0.0002	3.86	0.1067	
A^2	0.0078	1	0.0078	132.97	< 0.0001	
B^2	0.0003	1	0.0003	4.40	0.0901	
C^2	0.0009	1	0.0009	15.87	0.0105	
Residual	0.0003	5	0.0001			
Lack of Fit	0.0002	3	0.0001	2.25	0.3224	not significant
Pure Error	0.0001	2	0.0000			
Cor Total	0.1293	14				

The polynomial equation in mathematical form was scrutinized to unveil the intricate relationships between the dependent variables. Within this equation, a positive coefficient reflects a synergistic contribution to the response, whereas a negative coefficient sign indicates an antagonistic effect. Moreover, the magnitude of the coefficient elucidates the strength of the independent variable's impact on the response. The equation for response 1 is presented below, showcasing a robust correlation with its corresponding values. This mathematical representation provides valuable insights into the dynamics of the system and underscores the interplay between the independent variables and the response.

$$RF\ (Berberine) = +0.5233 + 0.1162 * A + 0.0375 * B + 0.0113 * C + 0.0025 * AB + 0.000 * AC + 0.0075 * BC + 0.0458 * A2 + 0.0083 * B2 + 0.0158 * C2$$

The investigation incorporated a graphical representation to elucidate the influence of distinct factors on responses, employing a surface plot. Specifically, a 3D response surface plot, alongside perturbation plots, was generated to explore the interplay among the volume of methanol, saturation time, and migration distance concerning the R_f value of berberine. The perturbation plots serve to delineate the envisaged model, thus augmenting our comprehension of the examined procedure. Through this visual depiction, the variability of the response amidst perturbations in each factor from its reference value, while holding all other variables constant at their reference points, is illustrated. Notably, the steepness of the slope or curvature discernible in the plot denotes the sensitivity to a particular factor, thereby furnishing invaluable insights into the influence of individual variables on the overarching response. Furthermore, the construction of surface response plots facilitated the elucidation of the relationship between the designated

factors at different levels and the response under scrutiny. These plots effectively showcase the impact of methanol concentration in the mobile phase, saturation time, and migration distance, each maintained at low, medium, and high levels, on the R_f values of berberine (Fig.-2). The prediction versus actual graph within the Design of Experiments (DoE) juxtaposes projected outcomes against observed results, serving as a cornerstone for model validation. In our model, this graph distinctly illustrates the alignment of both predicted and actual values along a singular trajectory, as depicted in Fig.-2.

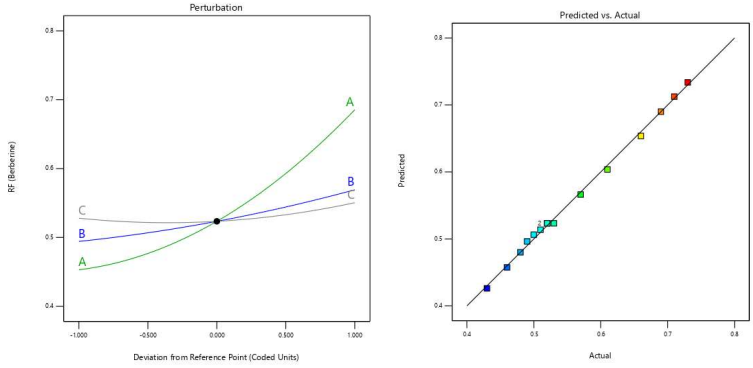

Fig.-2: The Effect of Each Factor, A, B, and C, on the R_f Value of Berberine, is Illustrated in Two Graphs: (a) Perturbation Graph and (b) Prediction Versus Actual Graph

Figure-3 vividly portrays the impact of independent factors on response Y1 (R_f Berberine). These findings emphasize the substantial influence wielded by the volume of methanol, saturation time, and migration distance on response Y1. Notably, as these factors increase, so does the response Y1, indicating a synergistic effect. This cooperative interaction among variables results in a more pronounced elevation of response Y1.

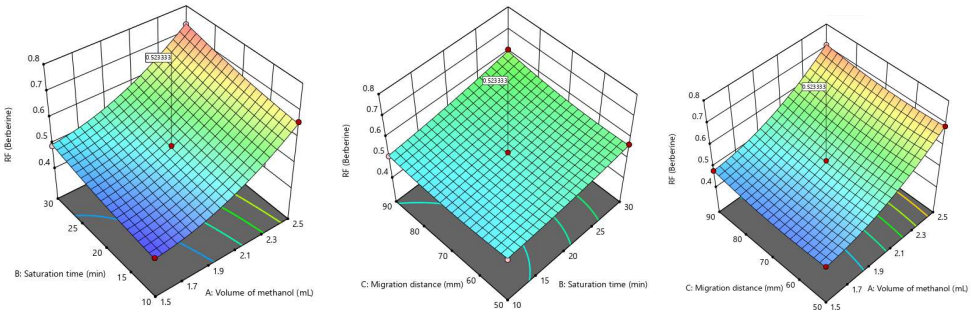

Fig.-3: Response Surface Plot Showing the Effect of Volume of Methanol (A), saturation Time (B), and Migration Distance (C) on R_f of Berberine

Utilizing Design-Expert software by Stat-Ease, Inc. (version 13.0), Each response generated favorable outcomes. From the array of improvement strategies offered by the software, conditions with desirability close to 1 were selected. These conditions were based on criteria ensuring the R_f value of berberine fell between 0.2 to 0.8, resulting in a desirability of 1. The optimal composition entailed methanol in the mobile phase, alongside ethyl acetate and water in a ratio of 2:7:1 v/v, a chamber saturation time of 20 minutes, and a migration distance of 70 mm. To validate the chromatographic conditions, six replicate injections of 500 ng per band of berberine were analyzed. The analysis revealed a difference of less than 5% between the observed and predicted R_f values of berberine, Verifying the precision and consistency of the selected conditions. Under optimal conditions, a remarkable chromatographic separation of berberine was achieved, as depicted in Fig.-4. This separation exhibited an R_f value of 0.52±0.01 for berberine, demonstrating the precision and efficacy of the method.

Characterization of Prepared Berberine/β-cyclodextrin Inclusion Complex Nanoparticles

The mean size and particle size distribution of the berberine/β-cyclodextrin inclusion complex nanoparticles were measured using the dynamic light scattering (DLS) method. The resulting nanoparticles exhibited an average particle size of 96.3±0.02 nm. The nanoformulation exhibited a uniform and narrow particle size

distribution., with a polydispersity index (PDI) value of 0.163±0.52. This indicates a high degree of uniformity and consistency, as PDI values below 0.9 are considered favorable.

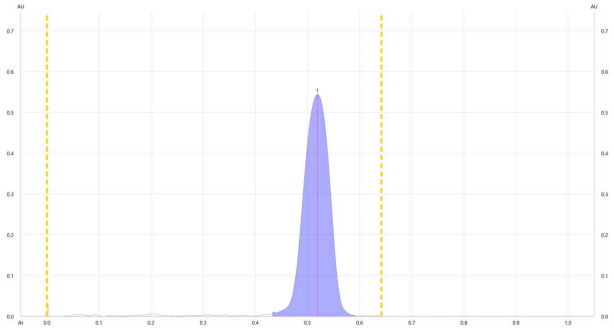

Fig.-4: Chromatogram of Berberine Containing 500 ng/Band using Proposed HPTLC Method

The zeta potential, an essential parameter for assessing the stability of nanoparticulate systems, was recorded at -23.6 mV. These zeta potential values suggest that the nanoparticles possess good stability. Additionally, our assessment of the free and total drug content in the nanoformulation showed remarkable encapsulation efficiency (EE) values. of 87.4±0.11%. The optimized levels of β-cyclodextrin likely led to the substantially increased EE percentages realized in the formulation.

Recovery Assay in Prepared Berberine/β-Cyclodextrin Inclusion Complex Nanoparticles

The proposed method was effectively utilized to determine berberine in the prepared β-cyclodextrin inclusion complex nanoparticles, with six replicate measurements conducted. As shown in Table-4, the results demonstrated excellent recoveries, ranging from 97.1% to 98.5%.

Table-4: Recovery Assay of the proposed method from prepared β-cyclodextrin inclusion complex nanoparticles.

Drug	Excess drug added to analytes (%)	Spiked (ng/band)	Found (ng/band)	Recovery (%)
Berberine	50	400	394±0.08	98.5
	100	600	583±0.12	97.1
	150	800	778.1±0.03	97.2

The quantitative estimation revealed a prominent peak for berberine, emphasizing the method's exceptional performance regarding sensitivity, accuracy, and precision. This guarantees the dependability of the results obtained, as demonstrated in Fig.-5.

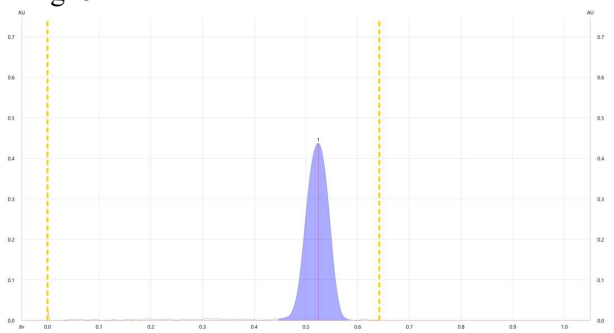

Fig.-5: HPTLC Chromatogram of Berberine in β-cyclodextrin Inclusion Complex Nanoparticles

Method Validation

To ensure the optimized HPTLC method is suitable for its intended application, we conducted thorough validation procedures in accordance with ICH Q2 (R1) standards. Table-5 summarizes the validation parameters for the proposed HPTLC method, all of which comply with the standard limits outlined in the ICH guidelines.

Linearity was established over a range of 200 to 1000 ng/band for berberine, yielding a correlation coefficient of 0.999. The Limit of Detection (LOD) and Limit of Quantification (LOQ), determined using linear regression data from the calibration curve, were 58.24 ng/band and 171.4 ng/band, respectively.

Precision evaluations for both intraday and interday assessments showed a percent Relative Standard Deviation (% RSD) below 2%. Robustness studies, involving slight modifications in chromatographic conditions such as mobile phase volume, migration distance, and saturation duration, also revealed a % RSD below 2%.

Table-5: Summary of Validation Parameters

S. No.	Validation parameters	Berberine
1	Linearity	
	Linearity range (ng/band)	200-1000
	Correlation-coefficient	<0.999
2	LOD (ng/band)	58.24
	LOQ(ng/band)	171.4
3	Precision	
	Intra-day (%RSD)	0.54
	Inter-day (%RSD)	0.85
4	Robustness	
	Mobile phase volume (%RSD)	0.67
	Migration distance (%RSD)	0.30
	Duration of saturation (%RSD)	0.87
5	Accuracy	
	50% recovery	99.41±0.11
	100% recovery	100.1±0.38
	150% recovery	98.41±0.41
6	Solution stability (%RSD)	0.63

The accuracy of the method, quantified by the recovery percentage, indicates the degree of concordance between the measured values and the true values. Experimental results at concentrations of 50%, 100%, and 150% demonstrated a high degree of precision for the newly developed method. A solution stability study showed no significant change in the peak area of berberine when the sample was analyzed after 48 hours, with a % RSD of 0.63. These findings indicate that the solution remained stable throughout the evaluation period.

CONCLUSION

The current research successfully developed and validated a High-Performance Thin-Layer Chromatography (HPTLC) method for the quantification of berberine, and effectively utilized this method to quantify berberine in cyclodextrin inclusion complexes. The method optimization, aided by the Box-Behnken Design (BBD), identified critical chromatographic factors and achieved a robust analytical procedure. The optimized HPTLC conditions, using a mobile phase of ethyl acetate, methanol, and water (7:2:1 v/v), ensured precise berberine separation, demonstrating an R_f value of 0.52 ± 0.01. Characterization of berberine/β-cyclodextrin inclusion complex nanoparticles revealed a mean particle size of 96.3 ± 0.02 nm, with a polydispersity index of 0.163 ± 0.52 and a zeta potential of -23.6 mV, indicating good stability and uniformity. The encapsulation efficiency was high at 87.4 ± 0.11%, underscoring the efficacy of the inclusion process. The method validation, conforming to ICH guidelines, affirmed the method's linearity, precision, accuracy, robustness, and solution stability. Linearity was confirmed within the 200-1000 ng/band range with a correlation coefficient of 0.999. Precision, both intra-day, and inter-day, showed % RSD below 2%, and accuracy testing exhibited recoveries between 97.1% to 98.5%, ensuring the method's reliability. In summary, this validated HPTLC method provides a reliable, accurate, and efficient tool for quantifying berberine in cyclodextrin inclusion complexes, with potential applications in quality control and formulation development within the pharmaceutical industry.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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