QbD-Driven Development and Validation of an Optimized HPTLC Method for Simultaneous Estimation of Berberine and Conessine

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Abstract

Berberine, an isoquinoline-derivative alkaloid from *Berberis aristata*, and conessine, an alkaloid found in the stem bark of *Holarrhena antidysenterica*, have long been used in traditional medicine to treat gastrointestinal and reproductive issues. This study focused on developing a high-performance thin-layer chromatography (HPTLC) method for the simultaneous quantification of berberine and conessine, optimized using a Quality by Design (QbD) approach. The method development involved the systematic optimization of critical method parameters (CMPs) such as mobile phase ratio, saturation time, distance travelled, and derivatizing agent concentration, using a Box-Behnken design. The critical analytical attributes (CAAs) evaluated included peak area and retardation factor as indicators of method robustness. Optimal chromatographic separation was achieved with a mobile phase of ethyl acetate, methanol, and diethyl amine in a ratio of 6.5:1.0:0.3 v/v on Silica gel 60GF 254 plates. Berberine and conessine were detected densitometrically at 350 nm and 620 nm, respectively, with Rf values of 0.22 and 0.85.

The method was validated as per the ICH recommended conditions, which revealed high degree of linearity, accuracy, precision, sensitivity and robustness. The method was demonstrated to be simple, fast, accurate, resilient, and exact. Also, the method was applied for the estimation of berberine and conessine in inhouse formulations, which indicated no significant change in the retention time. In a nutshell, the studies demonstrated successful development of the HPTLC method for simultaneous estimation of berberine and conessine with improved understanding of the relationship among the influential variables for enhancing the method performance.

INTRODUCTION

BERBERINE

The naturally occurring isoquinoline alkaloid berberine has drawn a lot of interest from pharmacologists and herbal medicine practitioners [1]. Usually isolated from Berberis aristata, a member of the Berberidaceae family of medicinal plants, it is also referred to as daruharidra. Due to its extraordinary therapeutic qualities, berberine has a long history of traditional use in many cultures, most notably in Chinese and Ayurvedic medicine [2–3]. Numerous pharmacological effects, including as antibacterial, anti-inflammatory, antiprotozoal, and antioxidant qualities, are exhibited by this alkaloid [4–5]. The main reason for its historical use has been its effectiveness in treating gastrointestinal conditions including diarrhoea and dysentery. Since the 1950s, berberine has been used as a non-prescription medication in China to treat diarrhoea brought on by a variety of etiologies[6–7].

Conessine is a natural steroidal alkaloid found in certain plants, particularly in the roots of *Holarrhena antidysenterica*, commonly known as Kurchi. Historically, conessine has been used in traditional medicine for various purposes, including the treatment of diarrhea and other gastrointestinal disorders [8–10]. It holds a prominent place in traditional Ayurvedic medicine, especially for the treatment of amoebic dysentery. It is valued for its antimicrobial, anti-inflammatory, and analgesic properties [11–12].

Both conessine and berberine exhibit antimicrobial activity against a range of pathogens, including bacteria and parasites that commonly cause gastrointestinal infections. Both compounds have demonstrated antidiarrheal, antimicrobial, and anti-inflammatory effects. Marketed formulation namely biquinol consist of both conessine and berberine.

In light of the widespread uses berberine and conessine and their impact on human health, it has become necessary to develop specific, sensitive, accurate and precise analytical method. Several HPLC, HPTLC and HPLC-MS methods have been developed for single component analysis of conessine [13–19] and berberine respectively [20–26]. However, there is not a single method available for simultaneous estimation of berberine and conessine.

Despite the available literature on estimating berberine and conessine using liquid chromatographic methods, effectiveness and reliability may face challenges due to high variability and inconsistent method performance. These variable results can be attributed to multiple factors, including mobile phase composition, chromatographic conditions, stationary phase, wavelength of analysis, and derivatizing agents.

In this context, method validation as per the recommended ICH guidelines does not provide much flexibility and reliability in variations in analytical method. As a result, the implementation of Quality by Design (QbD) principles for analytical method development has gained popularity for achieving high robustness and enhanced method performance [27]. The AQbD approach enables a science and risk-based understanding of the primary sources of variability, followed by the identification of Critical Method Parameters (CMPs) through risk assessment and factor screening studies. This process identifies high-risk variables that critically impact analytical performance, which are then optimized using suitable experimental designs to enhance method performance[28–29]. Over the past few decades, literature reports on various drugs have highlighted the substantial benefits of the AQbD approach in developing analytical methods for drug substances, impurities, and degradation products in an effective and cost-efficient manner [30–32].

Hence attempt was made to develop and validate analytical method for the simultaneous estimation of berberine and conessine by employing analytical QbD principles for estimation of berberine and conessine in bulk drug and pharmaceutical formulations.

In the present study, box–behnken design (BBD) was used to optimize the chromatographic conditions of the HPTLC method. Box– Behnken design was selected due to its flexibility to change, add, or delete any parameter at any time when our experiment is ongoing. The aim of this work was to develop and validate a quality by design-based efficient method using HPTLC.

EXPERIMENTAL

2.1 Chemical and Reagents:

Standards and reagents

Berberine and conessine were purchased from Yucca enterprises Mumbai. The commercially available tablet formulation of biquinol was used for assay. Ethyl acetate, methanol and diethylamine of analytical reagent grade was purchased from M/s SD Fine Chemicals, Mumbai, India. Silica gel GF TLC plates were purchased from merck pvt ltd. All other chemicals and reagents were purchased from loba chemie pvt ltd.

2.2 Instrumentation:

Methanol was used for the prewashing of HPTLC plates and activated by using an oven at 120°C for 10 min, prior to chromatography. The development was carried out in a 20 cm × 10 cm twin-trough glass chamber using Ethyl

Acetate: Methanol: diethylamine (6.5:1:0.3 v/v) as the mobile phase, after saturation of the chamber with mobile phase vapor for 10 min; the development distance was 80 mm. After completion of the development, the chromatography plates were dried with the help of an air dryer, and a CAMAG (Muttenz, Switzerland) HPTLC system – containing a CAMAG Linomat sample applicator, a Hamilton syringe (Bonaduz, Switzerland), and a CAMAG TLC Scanner with winCATS software – was used for the current analytical study.

2.2.1 Preparation of Stock Solution:

25 mg standard berberine was weighed and transferred to 50 ml volumetric flask and methanol was added to 50 ml. 100 mg of conessine was weighed and dissolved in 10 ml methanol.1 ml from standard berberine of 500ug/ml and 1 ml from standard conessine of 10000ug/ml was transferred to 10 ml volumetric flask and diluted up to 10 ml with methanol to get 50ug/ml of berberine and 1000ug/ml of conessine which makes it working standard solution for simultaneous estimation of berberine and conessine.

2.2.2 Selection of Analytical Wavelength:

After chromatographic development, the bands were scanned over the range 200–800 nm which was used for detection analytes during method development and validation. The structures of berberine and conessine are presented in Fig. 1.

2.3 Method development as per experimental design:

In the beginning of risk assessment plan, Ishikawa fishbone diagram was constructed using Microsoft Excel to structure the risk operation plan for the critical factor affecting the method critical analytical attributes (CAAs).

Mobile phase is one of the crucial parameter in method development hence various composition of mobile phases were tried to get resolution of berberine and conessine which are presented in Table 1S.

Based on the preliminary results of mobile phase composition, four-factors were used as Critical method parameters namely Mobile phase composition, saturation time, development of plate and derivatizing agents and 27-run regular 4-factorial design was implemented for factor screening studies to know the critical method parameters/critical process parameters which mostly affect the CAAs(area and Rf). The design matrix enrolled considered factors and their i.e., low (-1), intermediate (0) and high (+ 1) levels suggested by the software, as shown in Table 1. A standard concentration 50 µg/mL and 1000 µg/mL of berberine and conessine respectively were used for all experimental runs, which were analyzed for method CAAs, i.e. peak area and retardation factor. The experimental design runs were analyzed for the influence of the study factors on the CAAs. A summarized design matrix consists of 27 experimental runs as per the Box–Behnken design (Table 1). Translation of coding in actual units for the 27 experimental trials as per box–behnken design matrix is given in Table 2S. All the results were assessed using statistical software Design–Expert® version 11.

Table 1
Design matrix as per BBD for optimization of HPTLC method for simultaneous estimation of berberine and conessine

Run	Factor 1 (A:mobile phase v/v)	Factor 2 (B:saturation time mins)	Factor 3 (C:distance travelled mm)	Factor4 (D: anisalehyde concentration)
1	-1	-1	-1	1
2	-1	-1	-1	-1
3	0	1	0	0
4	1	-1	1	-1
5	0	-1	0	0
6	-1	1	1	1
7	1	1	1	1
8	-1	0	0	0
9	0	0	0	1
10	-1	-1	1	1
11	1	1	-1	-1
12	-1	1	1	-1
13	1	-1	-1	-1
14	0	0	1	0
15	1	1	-1	1
16	1	-1	-1	1
17	0	0	-1	0
18	1	1	1	-1
19	0	0	0	0
20	-1	-1	1	-1
21	-1	1	-1	1
22	-1	1	-1	-1
23	1	0	0	0
24	0	0	0	0
25	1	-1	1	1
26	0	0	0	0
27	0	0	0	2

2.4. HPTLC method optimization

The HPTLC chromatographic conditions were optimized on the basis of results of BBD. The reference standards of berberine and conessine were individually weighed and dissolved in methanol to get the standard solutions having concentration 10000ppm. Stock solutions was diluted suitably to obtain the working standard 50 ppm and 1000 ppm of berberine and conessine.

Table 2: Result of BBD study for comparison of Rf Value between the predicted value and observed values By DoE Software

Response	Mean	Predicted	Observed
Rf of berberine	0.197719	0.197719	0.28
Ref of conessine	0.832561	0.832561	0.80

Camag linomat V sample applicator (Switzerland), was used to inject various amounts of working standards for the aforementioned marker compounds in the band form (6 mm wide) using a Hamilton microliter syringe (100µl). The dimension of slit was 5 mm x 0.45 mm. Appropriate volumes of standard solutions were separately loaded on precoated TLC plates of silica gel 60GF254 (Merck) to get graded amounts in the range of 100-800ng/band for berberine and 2000–16000 ng/band for conessine. The mobile phase of ethyl acetate: methanol: diethylamine 6.5:1:0.3 v/v was used as optimized mobile phase based on results of BBD. Horizontal elution of 20min was done followed in the chromatography process. Plate was removed and kept for drying in over 100 ℃ for 15 min and observed in CAMAG TLC Visualizer at 350 nm.

After Post Derivatization the HPTLC plate was derivatized using Anisaldehyde (1%) sulfuric acid for quantification of Conessine. The derivatized plate was then scanned at 620 nm using CAMAG TLC densitometric Scanner 3 incorporated with winCATS 1.4.8 programming. Plate was dried after development and visualization was performed using CAMAG TLC Scanner4 at wavelength 350nm for berberine. The plate was further derivatized using derivatizing agent 1% Anisaldehyde sulfuric acid for detection of conessine at 620nm.

2.5 Validation of the HPTLC Method

The optimized HPTLC method was validated for linearity, accuracy, precision, robustness, specificity and system suitability using ICHQ2R1 guideline [33].

a. Linearity

As per ICH guidelines Q2 (R1), for the establishment of linearity, a minimum of 5 concentrations is recommended. It was determined by plotting a graph of peak area v/s concentration of standards to obtain correlation coefficient (R2) and equation of the line. 50 ppm and 1000 ppm of berberine and conessine were used as working standard solution. The different volumes of standard stock solution, 4, 8, 10, 16,24 and 30µL, were spotted on HPTLC plate (10 × 10 cm) for berberine and Conessine to get concentration of 100-800ng/spot for Berberine and 2000-16000ng/spot for conessine. The peak areas of all concentrations of linearity were measured with CAMAG HPTLC Software vision CATS for both markers Berberine and Conessine and spots were scanned By TLC SCANNER3. The method was used for quantification of biomarkers berberine and conessine from the inhouse formulation containing standardized extract of berberine and conessine.

b. Precision

Interday and intraday precision of the analytical method was determined at three concentrations of quality control. The three quality control concentrations at LQC, MQC, and HQC for 200, 400, and 600 ng/band for berberine and 4000, 8000 and 12000 ng/band for conessine, respectively. The precision was expressed as % RSD at each concentration of quality control samples. The intra-day precision of the marker compounds was assessed using six replicates of quality control samples on the same day. The inter-day or reproducibility parameter was evaluated by analysing QC sample for three consecutive days under the same set of experimental conditions. The results of precision are expressed in terms of the % RSD. (Table 3)

Table 3
Intraday and interday precision of berberine and conesssine

	Intraday Precision		**Intraday Precision**	
QC samples	Berberine Concentration ng/band	Conessine Concentration ng/band	Berberine Concentration ng/band	Conessine Concentration ng/band
LQC	204.06	3997.51	199.88	4108.46
	199.84	4040.08	187.21	3995.59
	204.56	4153.75	190.79	4132.22
Mean concentration	202.82	4063.78	192.63	4078.76
RSD	1.64%	1.98%	1.31%	1.78%
MQC	383.73	7845.64	398.13	8024.11
	389.76	8127.25	394.79	7913.07
	381.76	8038.60	404.13	8018.98
	385.09	8003.83	399.2	7985.39
RSD	1.08%	1.79%	1.65%	0.78%
HQC	582.82	12120.02	602.16	11811.61
	587.19	11906.53	591.30	11750.64
	595.91	11741.93	603.53	12126.53
Mean concentration	588.64	11922.83	598.99	11897.26
RSD	1.13%	1.58%	1.44%	1.68%

c. Accuracy

Accuracy study was performed for markers berberine and conessine by % recovery method. The stock solution was spiked in the formulation having known concentration of berberine and conessine at different levels viz. 50, 100 and 150% considering all biomarker. The analysis of all samples by HPTLC method was carried out and

percent recovery of each biomarker was calculated to confirm accuracy of the analytical method. Berberine solution having concentration 500 µg/ml standard solution spiked with 50% (250 µg/ml), 100% (500 µg/ml) and 150% (750 µg/ml) of additional amount of standard berberine. 8 µl volume of each solution was loaded on HPTLC plate to get amount of berberine as 600, 800 and 1000 ng/spot. Conessine having concentration 5000 µg/ml was used as standard solution which is spiked with 50% (2500 µg/ml), 100% (5000 µg/ml) and 150% (7500 µg/ml) of additional amount of standard conessine. Further, SD, %RSD and SEM were calculated to check the accuracy of data within the specified limit.

d. Robustness

Robustness parameter was assessed by slight modification in the chromatographic conditions viz mobile phase composition, mobile phase saturation time and wavelength of analysis. The change in area and Rf value of marker compounds was monitored. The final results were examined by calculation of % RSD of concentration.

e. Limit of detection and limit of quantitation

The LOD and LOQ were determined from the slope (S) of the linearity plot and standard deviation of the response to the blank sample (σ) as per the formulae enlisted in Eq. (1).

LOD = 3.3σ/S LOQ = 10 σ/S (Eq. 1)

f. Specificity

Inhouse formulation was formulated containing phytoconstituents berberine, conessine, piperine and quercetin. Specificity of berberine and conessine in presence of other phytoconstituents piperine and quercetin was evaluated. The specificity parameter was evaluated by comparing the chromatograms of the solvent (blank chromatogram) and marketed formulation with the marker compounds. A methanolic solution of standard Berberine and conessine was developed with diluent methanol and compared against blank solution.

2.6 Assay of inhouse Formulation:

Inhouse formulation was prepared by pharmacognosy department which was analysed using validated HPTLC method. Formulation containing *B. aristata* extract (500 mg), *H. antidysenterica* extract (70 mg), Piperine (2.5 mg) and Quercetin (2.5 mg) was used for the assay and amount of berberine and conessine was determined. 5 capsules were weighed accurately and granules were crushed using mortar and pestle. This powder was transferred to 50 ml volumetric flask and diluted with methanol 30ml and sonicated for 15 mins or till dissolution of both the extracts as both the markers are soluble in methanol and the volume was made up to the mark using methanol, solution was then filtered through Whatmann filter paper. (Solution I.) Solution I (1ml) was transferred to 10 ml volumetric flask and made up the volume to the mark with methanol (Solution II). Solution II was then analysed by HPTLC using the analytical method as described in section 2.5 to get content of berberine and conessine. The results are expressed as Mean mg/capsule ± SD.

RESULTS AND DISCUSSION

3.1 Preliminary method development studies

The preliminary studies were carried out as per the literature reports for developing the HPTLC method for estimation of berberine and conessine. As the majority of the reported methods describe the method for single component analysis which includes, the use of complex mobile phase composition, variable flow rate (isocratic/gradient elution) and temperature setting, etc., for chromatographic separation of berberine and conessine individually, it was envisaged to prepare a simple, sensitive and cost-effective HPTLC method for simultaneous estimation of berberine and conessine. Initially, various combinations of mobile phase were attempted by employing toluene, ethyl acetate, formic acid, diethylamine, chloroform and methyl chloride. The preliminary studies suggested the selection of ethyl acetate, diethylamine and methanol as solvents for the mobile phase, owing to better resolution of berberine and conessine along with adequate peak symmetry.

3.2 Optimization of Data Analysis

In optimization, data analysis was carried out by selecting a quadratic model for detection of retardation factor and peak area with Design–Expert® software version 11. The effect of Mobile phase composition and saturation time on analysis of berberine and conessine are given in Figs. 2 and 4. The 3D response surface plot observed for CAAs is shown in Figs. 3 and 5. The graphs shown in Figs. 3 and 5 indicates a correlation between the various factors and confirmed its significant effect on simultaneous estimation of berberine and conessine. To be more specific it can be commented that as the mobile phase composition changes the Rf value decreases. It was observed that as the saturation time and distance travelled by solvent increases the Rf value of conessine increases. The Rf value is directly proportional to the saturation time and distance travelled by solvent.

Results of BBD study and correlation between predicted and observed RF value of berberine and conessine are shown in Table 2. It is confirmed that the model was statistically significant in its prediction of Rf, as portrayed by a probability value of less than 0.05. All quadratic terms were found to be significant for the responses of RF and peak area. The numerical optimization suggested optimized conditions having a band length of 0.4 mm, a saturation time of 10 min, and a wavelength of 266 nm shown in Fig. 6.

Interactive effects of critical factors on simultaneous estimation of berberine and conessine was shown in Fig. 6. Linear increase in the Rf value was observed due to interactive effect of the saturation time and mobile phase. At higher level of the both factors, the Rf value was found to be in the range of 0.19 to 0.25 for berberine and 0.80 to 0.86 for conessine and at the lower level of the factors it was found to be 0.1 to 0.23 for berberine and 0.73 to 0.81 for conessine. At 0 level the optimum results were obtained.

As per results of DOE experiment, Simultaneous analysis of Berberine and Conessine was obtained in predicted range with the mobile phase having composition Ethyl acetate: Methanol: Diethylamine in the ratio of 6.5:1:0.3 v/v with saturation time of 20mins and it was derivatized using 1% v/v anisaldehyde sulphuric acid reagent. The representative TLC image of optimized method is given in Fig. 1S.

3.3 Analytical Method Validation

The optimized HPTLC method was validated for linearity, accuracy (%recovery), precision, robustness, limit of detection (LOD), and limit of quantification (LOQ) according to the International Conference on Harmonization (ICH) guidelines.

a. Linearity:

The standard calibration curve was plotted in a concentration range of 100–800 ng per band and 2000–16000 ng per band for berberine and conessine, respectively. The used linearity range has shown adequate coefficient values (R^2 = 0.9967 and R^2 = 0.9986). The linear regression equations were found to be $y = -0.013x^2 + 32.695x + 4117.4$ for berberine and $y = -5E\text{-}05x^2 + 2.1224x - 551.87$ for conessine. Representative chromatograms of berberine and conessine are shown in Fig. 7 and responses of 3 sets of linearity are presented in Table 3S. The calibration curves for the linearity are shown in Fig. 8 for berberine(a) and conessine(b) respectively.

b. Specificity

Inhouse formulation was formulated containing phytoconstituents berberine, conessine, piperine and quercetin. Specificity of berberine and conessine in presence of other phytoconstituents piperine and quercetin was evaluated. All four phytoconstituents namely quercetin (Rf = 0.15), piperine (Rf = 0.90), berberine (Rf = 0.22) and conessine (Rf = 0.85) were separated and well resolved with no interference was observed from the other constituents (Fig. 9).

c. Precision

The precision was estimated at three different concentration levels of LQC (200ng/band), MQC (400 ng/band) and HQC (600 ng/band) for berberine and of LQC (4000ng/band), MQC (8000 ng/band) and HQC (12000 ng/band) for conessine to evaluate reproducibility. Intraday precision was assessed by analysizing quality control solutions on the same day in triplicates. Interday precision was carried out in the same conditions by same analyst during three different days. Representative chromatograms of quality control standard solutions are presented in Fig. 2S and 3S for berberine and conessine respectively. The %RSD values for berberine and conessine intra-day were found to be 1.08–1.64% and 1.58–1.98%, respectively. The %RSD values for berberine and conessine inter-day were found to be 1.31–1.65% and 0.78–1.78%, respectively (less than 2). Results are presented in Table 3.

d. Accuracy

Accuracy studied was performed for berberine and conessine

in terms of recovery. For this study, spiking was done on the drug substance and drug product. In the proposed method, accuracy was calculated by spiking standard drug in drug product. The calculated %recoveries against respective levels are mentioned in Table 4.

Table 4
Accuracy for Berberine and conessine by recovery method

Sample	Spike level	Amount of drug present in formulation (mg)	Amount of drug added (mg)	Amount of total drug recovered (mg)	Recovery (%)	Average recovery
Berberine	50%	500	250	751.0466	100.14	96.88
	100%	500	500	912.156	91.22	
	150%	500	750	1241.21	99.30	
Conessine	50%	5000	2500	7989.17	106.52	95.24
	100%	5000	5000	8902.58	89.03	
	150%	5000	7500	11270.03	90.16	

e. Robustness

The standard deviation of areas was calculated for each condition, and %RSD was less than 2%. The results of robustness studies are shown in Tables 5. Robustness study confirmed that the HPTLC method is robust and hence can give high degree of accuracy and precision. Results are presented in Table 5.

Table 5
Robustness study of berberine and conessine

Parameters	Robustness parameter composition	Berberine RSD%	Conessine RSD%
Mobile phase (6.5:1:0.3) v/v/v	5.7:1:0.3 v/v	1.79	1.79
	6.5:1:0.2 v/v	1.42	1.42
Wavelength (350nm)	348 nm v/v	0.12	0.81
	352nm v/v	0.17	1.06

f. Limit of Detection and Limit of Quantification

The LOD and LOQ of the developed method were found to be 57.29 and 171.88 ng per band for berberine and 486.19 and 1458.57 ng per band for conessine, respectively, indicating the sensitivity of the proposed method. The results of LOD and LOQ are shown in Table 6.

Table 6
summary of linear regression and validation data

Parameters	Berberine	Conessine
Concentration range (ng/band)	100-800ng/band	2000-16000ng/band
Correlation coefficient (r2) ± SD	0.9986	0.9988
LOD (ng/band)	57.29	486.19
LOQ (ng/band)	171.88	1458.57
Accuracy by% recovery ± SD	96.88	95.24
Precision		
Intraday % RSD	1.08–1.64%	1.58–1.98%
Interday precision % RSD	1.31–1.65%	0.78–1.78%

3.4 Method application in Assay of inhouse formulation

Inhouse formulation was formulated containing phytoconstituents berberine and conessine, piperine and quercetin. Analytical method was found to be specific for berberine and conessine in presence of other phytoconstituents piperine and quercetin. Figure 10 illustrates the chromatograms of berberine and conessine in inhouse formulation. All chromatograms revealed no significant change in the Rf value, of the berberine and conessine. Mean content of marker compounds berberine and conessine was found to be 15.5 mg and 2.8 mg respectively in capsule formulation (Table 7).

Table 7
Assay of inhouse formulation containing standardized alcoholic extracts of Roots of *Berberis aristata* and Bark of *Holerrena antidysentrica.*

sr.no	Extract	Marker	Mean content of marker in100 mg extract	Amount of extract in capsule in mg	Expectedmarker in 1capsulenmg	Mean content of marker (mg) in formulation	% content
1	*B. aristata*	berberine	3.2 mg	500 mg	16 mg	15.5 mg	96.87%w/w
2	*H.antidysentrica*	conessine	4.2 mg	70 mg	2.9 mg	2.8 mg	96.55%w/w

Discussion

This study effectively applied Quality by Design (QbD) principles to develop a High-Performance Thin-Layer Chromatography (HPTLC) method for berberine and conessine, resulting in enhanced robustness and performance. Key factors were identified through initial prioritization and screening, then optimized to improve method robustness. Response surface mapping provided a deeper understanding of the relationships and interactions between factors and their responses. Comprehensive validation confirmed the method's robustness, even under extreme variations in key variables. The method proved highly selective and precise for quantifying

berberine and conessine in formulation, maintaining consistent retardation factors, and ensuring specificity and selectivity for analysis purposes.

Conclusion

A straightforward, rapid, sensitive, and cost-effective analytical method has been successfully developed using a systematic Quality by Design (QbD) approach for the simultaneous estimation of berberine and conessine in pharmaceutical formulations. Through factor screening and optimization studies employing experimental designs, critical method attributes (CMPs) was identified, which facilitated understanding the relationship between CMPs and critical analytical attributes (CAAs). Various chromatographic variables like saturation time, mobile phase and distance travelled were simultaneously optimized by applying a useful experimental design tool i.e Box–Behnken design. The optimal chromatographic conditions were determined as part of the analytical design. In a nutshell, the studies unequivocally vouch the applicability and utility of science and risk-based QbD approach for developing an LC method of berberine and conessine, an approach that can be extrapolated to other drug substances too. Also the developed method can be transferred to bioanalytical setup for estimation of the berberine and conessine in pharmacokinetic and bioequivalent study samples.

Declarations

Competing Interests:

The authors declare that there is no conflict of interest with respect to the article.

Authors Contribution

Dr. Archana Naik guided the bioanalytical method development work and Rutuja Nagargoje and Sriniwas Yadav developed analytical method for estimation of berberine and conessine. Dr. Chhaya Gadgoli guided preparation of formulation containing standardized extract of berberine and conessine. All authors have read and agreed to publish manuscript.

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Figures

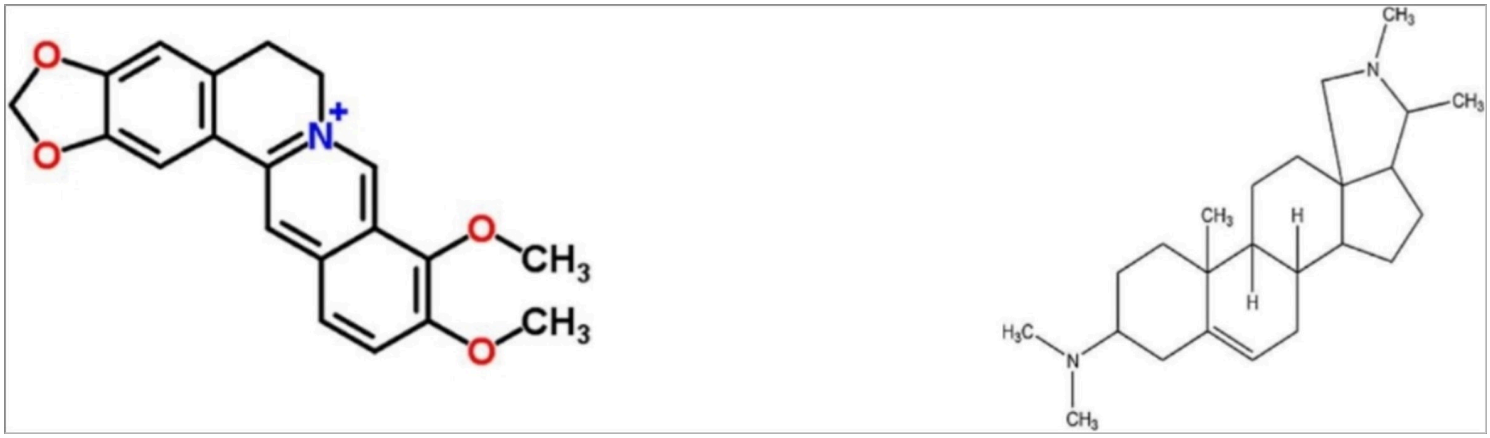

Figure 1

Structure of berberine (I) and conessine (II)

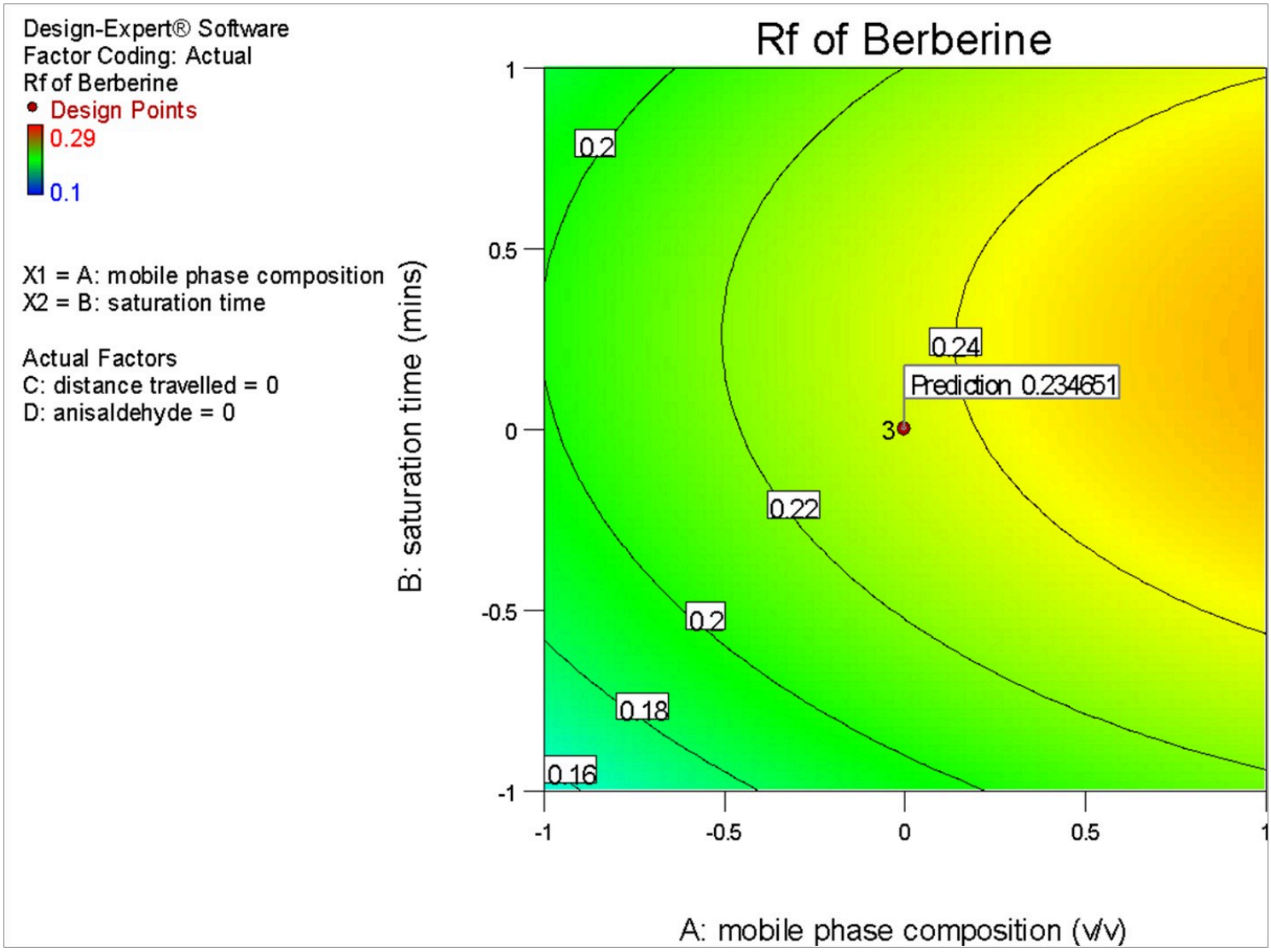

Figure 2

Prediction graph using Design expert software indicating effect of mobile phase composition and saturation time on Rf of berberine

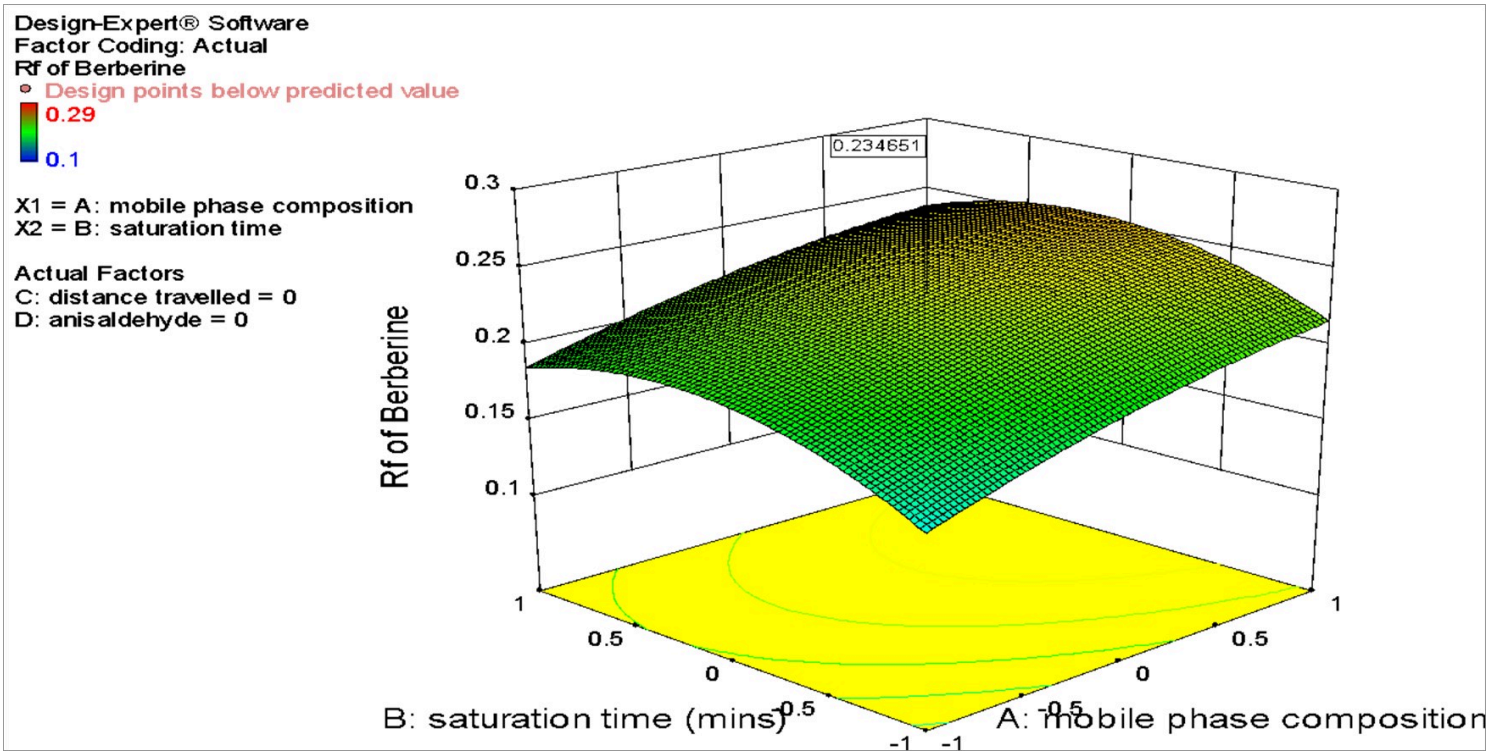

Figure 3

3D response surface plots of berberine showing (a): mobile phase v/v, (b): saturation time mins, (c): distance travelled mm (d): anisaldehyde on the RF value.

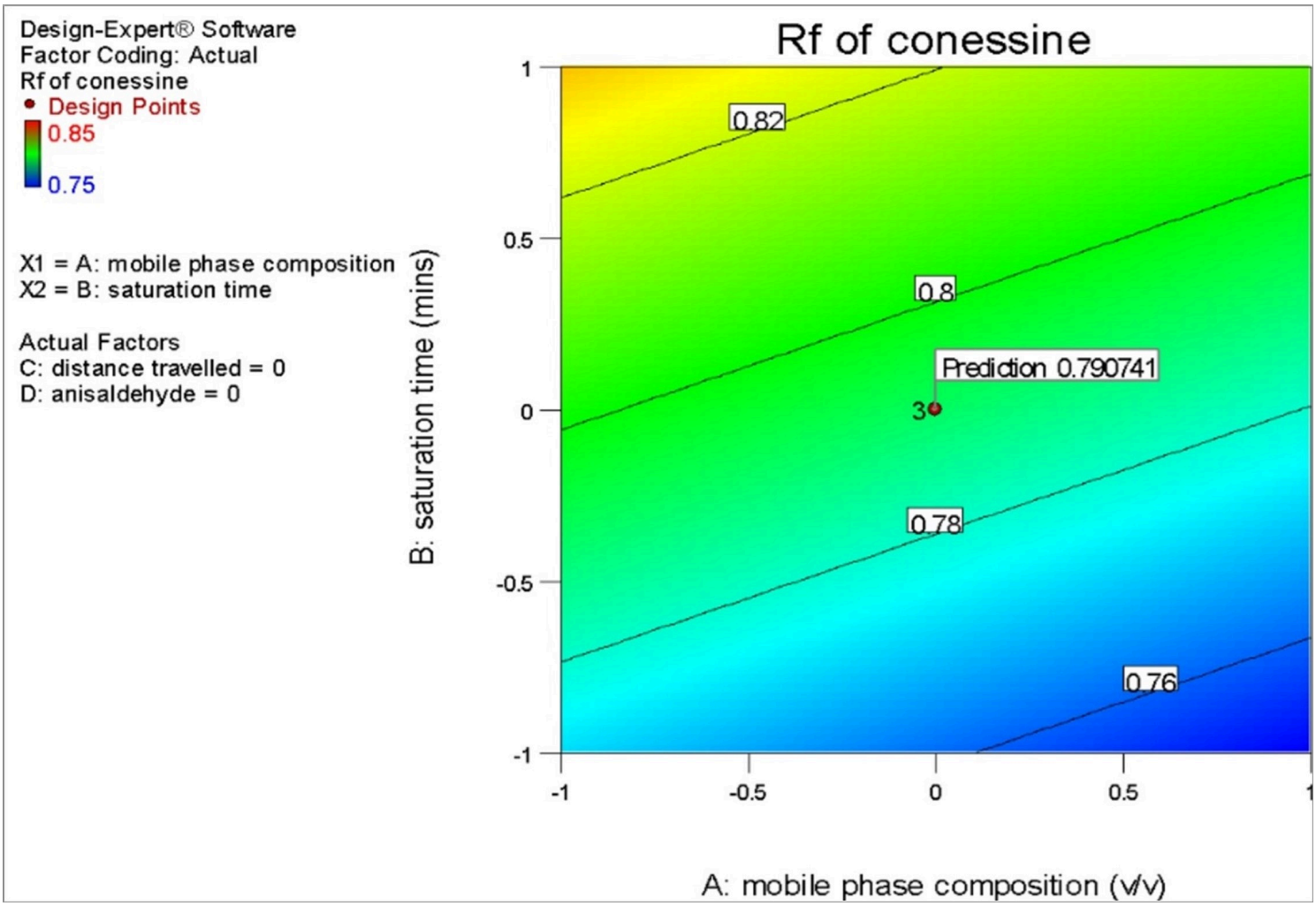

Figure 4

Prediction graph using Design expert software indicating effect of mobile phase composition and saturation time on Rf of Conessine

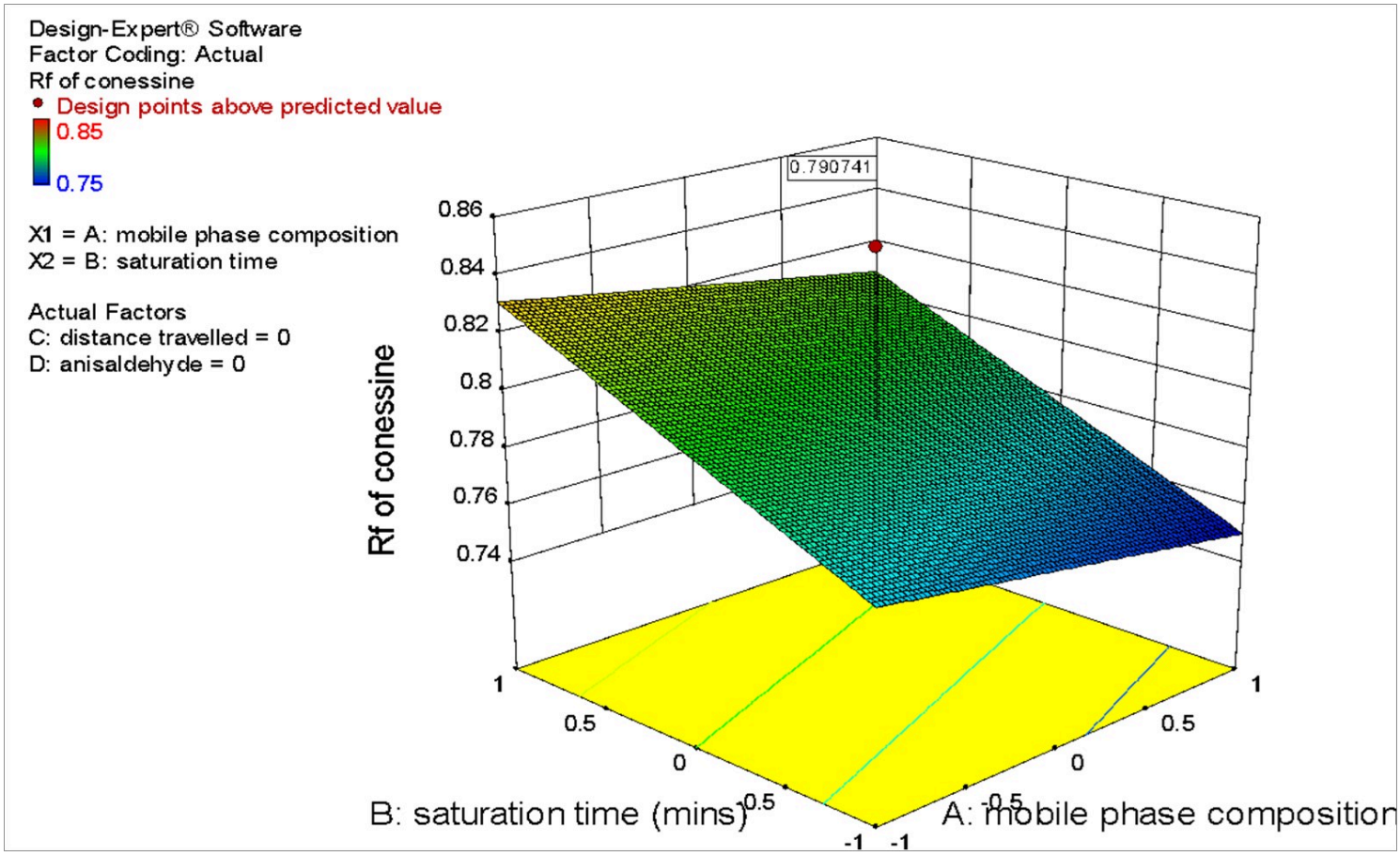

Figure 5

3D response surface plots of conessine showing (a): mobile phase v/v, (b): saturation time mins, (c): distance travelled mm (d): anisaldehyde on the peak

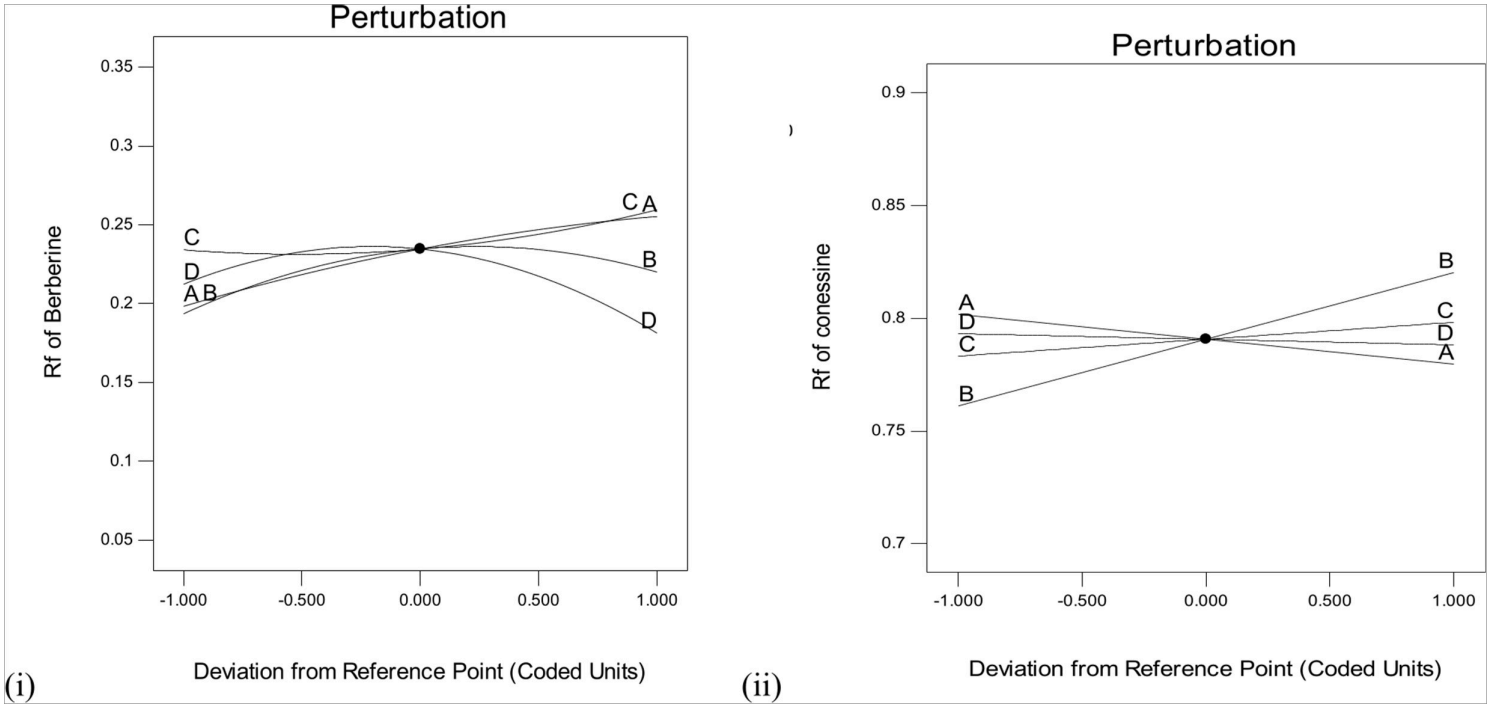

Figure 6

Interactive effect of A) mobile phase composition, B) saturation time C) distance travelledD) anisaldehyde on Rf of berberine (i) and conessine (ii).

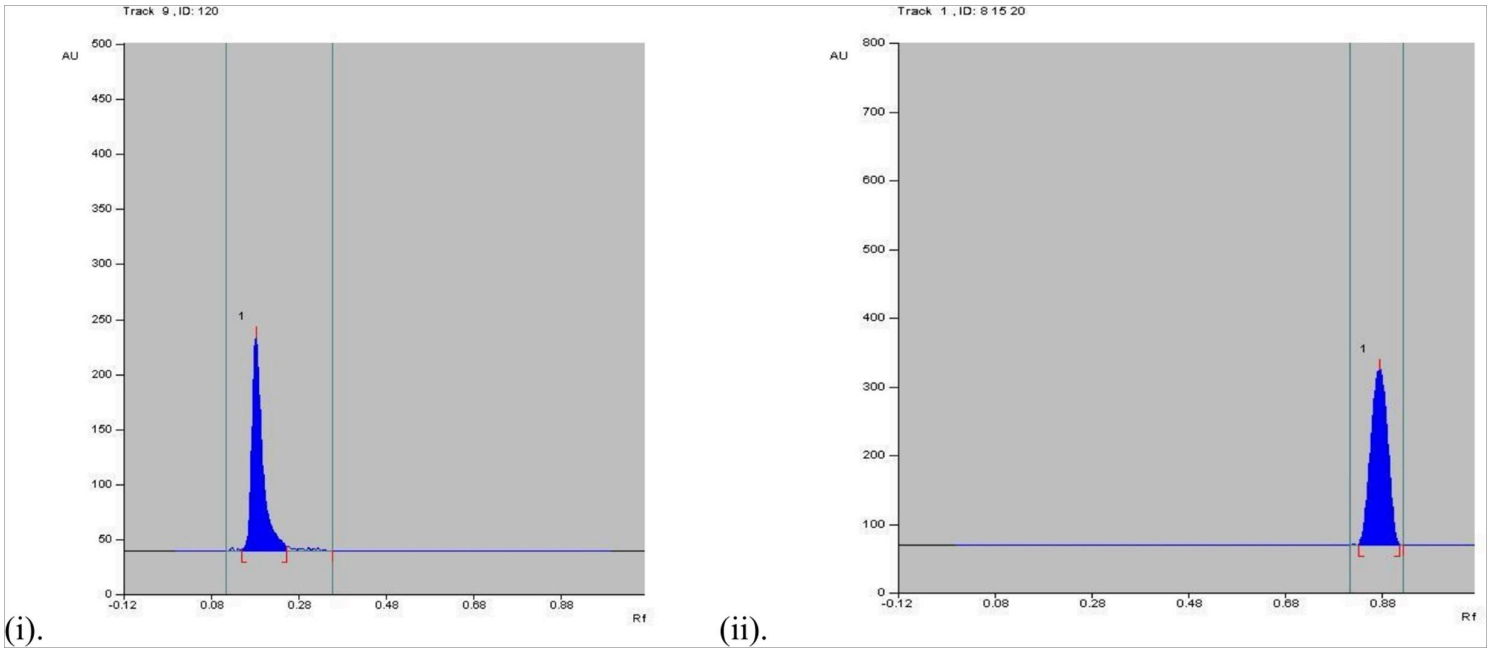

Figure 7

Representative HPTLC chromatogram of berberine(a) and conessine(b)

Mobile phase : ethyl acetate: methanol: diethyl amine (8.3:1.3:0.4v/v/v).

(a) Berberine : Visualization under UV 365 NM [quantification at 350 nm]

(b) Conessine : Visualization in visible light after derivatization with Anisaldehyde and Sulfuric acid [quantification at 620 nm]

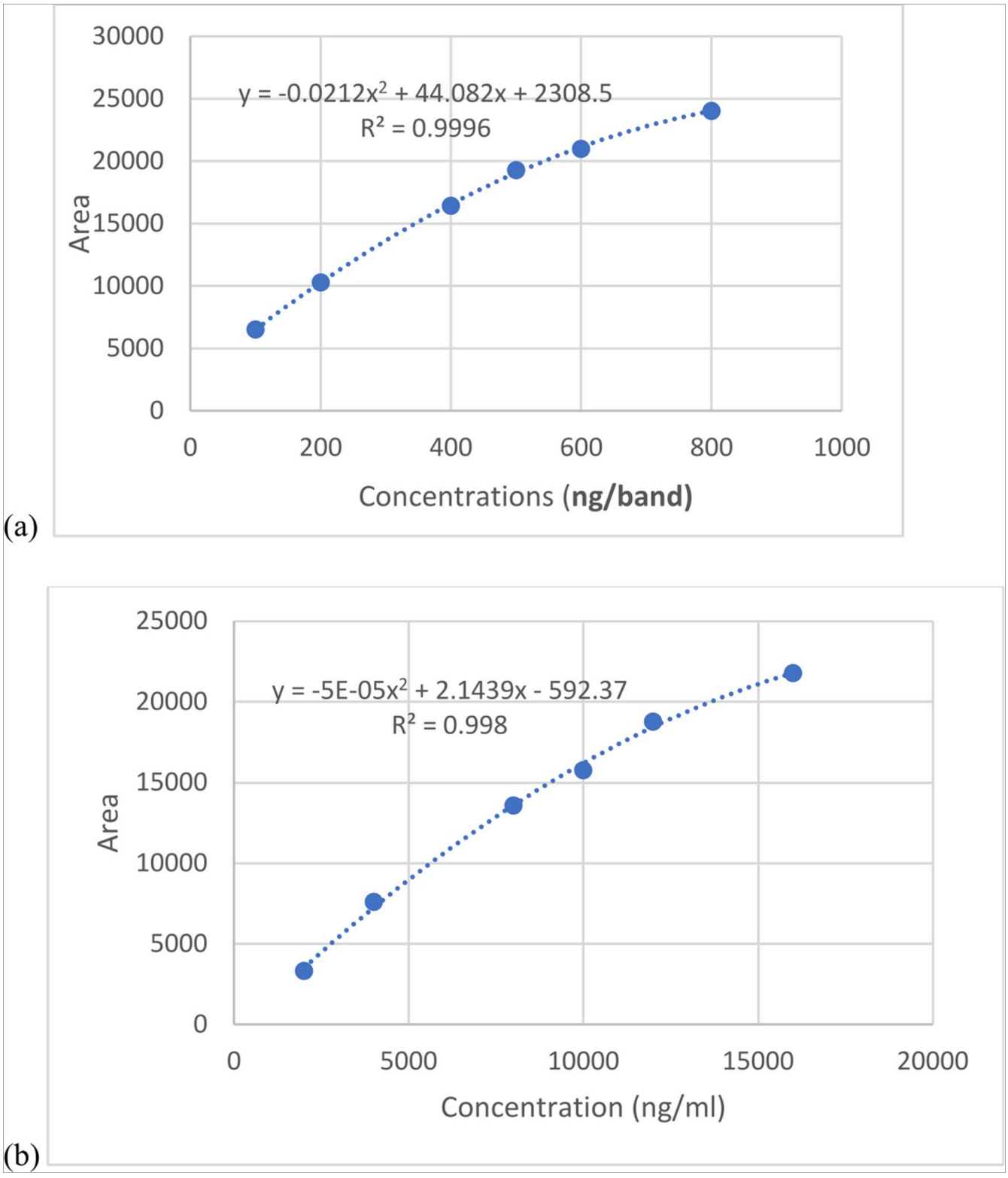

Figure 8

Calibration curve for Berberine (a) and Conessine (b)

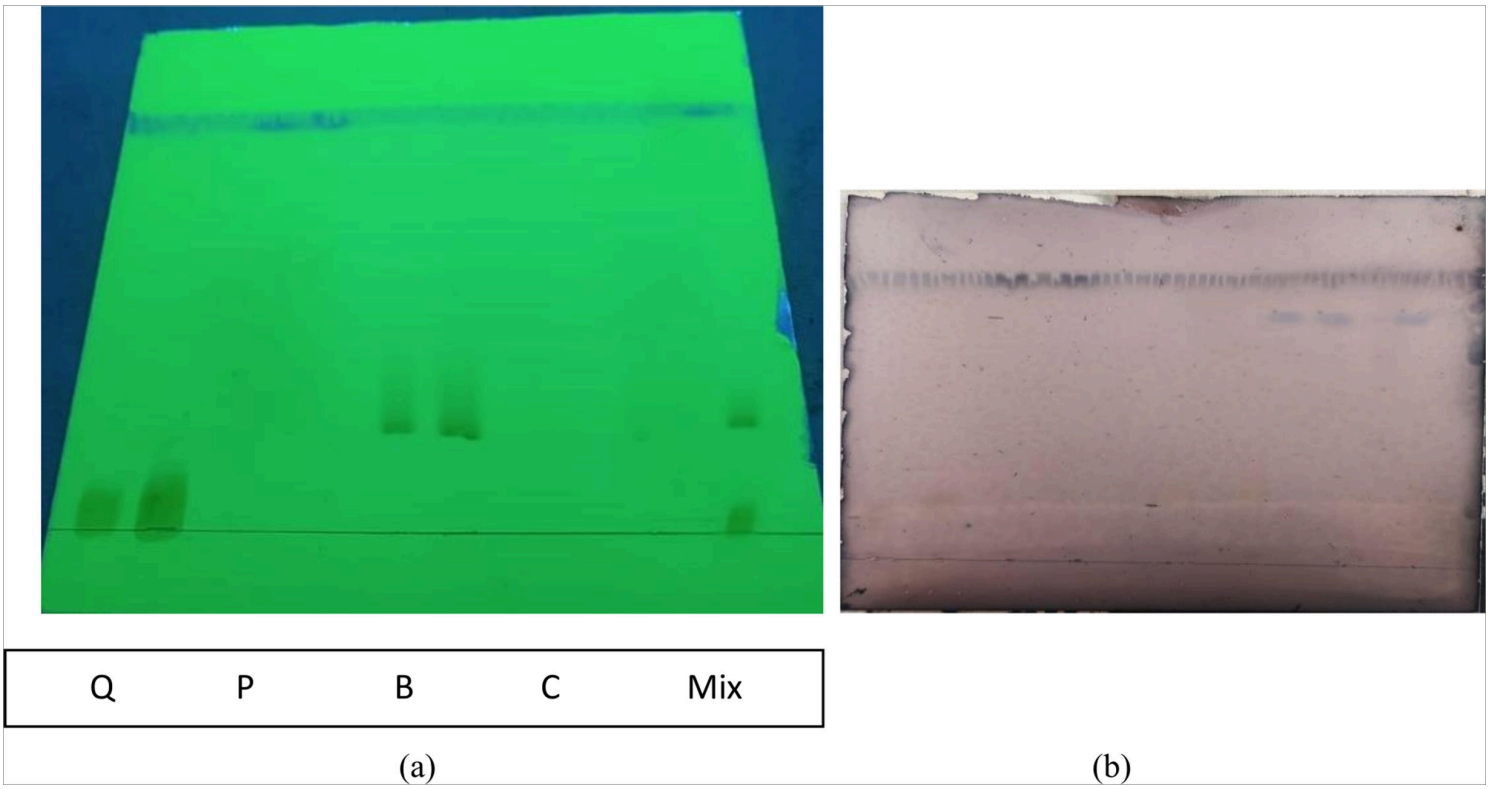

Figure 9

Specificity study for simultaneous estimation of berberine and conessine. **TLC represents separation of quercetin(Q), piperine(P), berberine(B) before derivatization and conessine(C) detection after derivatization with 1% anisaldehyde solution using mobile phase ethyl acetate: methanol: diethyl amine in the ratio (6.5:1:0.3v/v)**

(a) TLC showing selective analysis of berberine at R.f (0.23) visualization under UV light at 350nm

(b) TLC showing selective analysis of conessine at R.f (0.8) after derivatizing and visualization under visible light with Anisaldehyde 1% solution

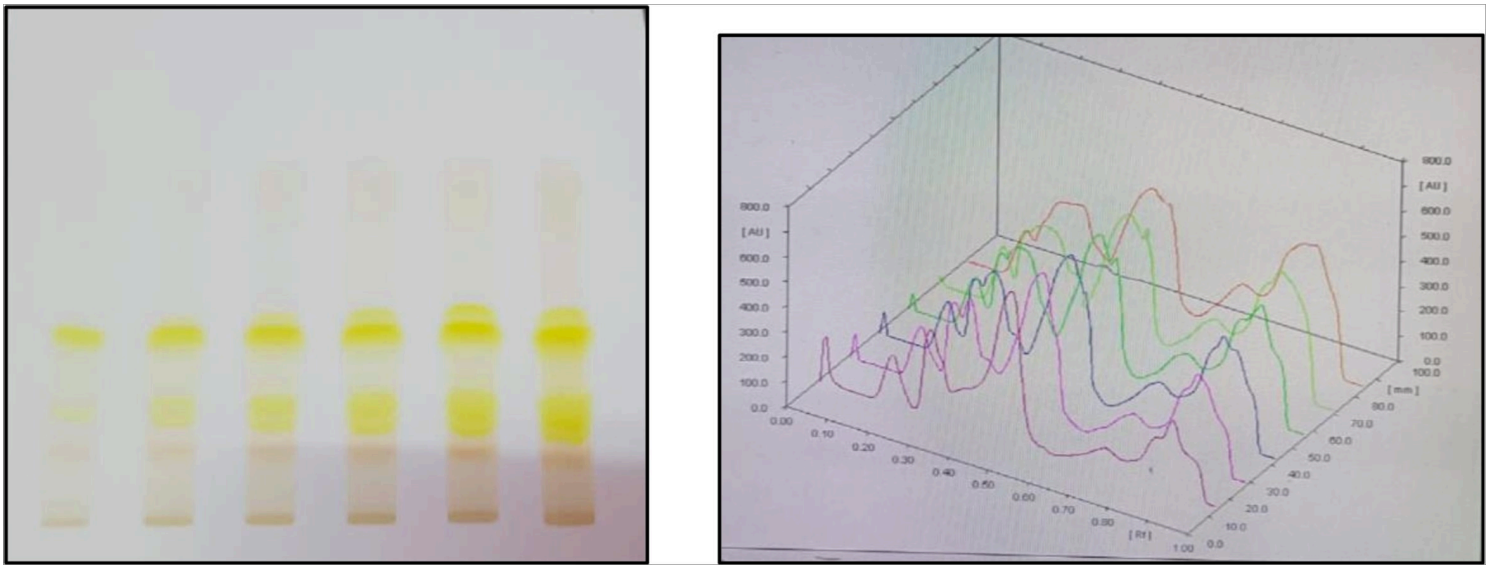

Figure 10

Application of Validated HPTLC method for assay of inhouse formulation containing standardized Alcoholic extracts of Roots of *Berberis aristata* and Bark of *Holerrena antidysentrica.*

TLC (a) and HPTLC densitometric analysis (b)

Supplementary Files

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