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Analytical Quality by Design Based UV-visible Spectrophotometer Method Development and Validation for Quantification of Chrysin in Bulk and Nanostructured Lipid Carriers

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ABSTRACT

A simple analytical UV-visible spectrophotometric method was developed and validated. For that central composite design (CCD) was used to optimize a UV-visible spectrophotometer method for analysis of chrysin (CS). The sample interval and scanning speed were selected as the critical method variables (CMV). The CCD produced the 13 runs for optimization of CMV. Based upon the obtained response results the optimum condition of sample speed and sample interval selected was -0.60 nm/sec and -0.51 nm, respectively. The calibration curve of CS was plotted for concentration range of 2-10 µg/ml. The method was validated for its linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) as per ICH Q2 (R1) guidelines. The developed method for CS was found to be linear in the concentration range of 2-10 µg/ml with high correlation coefficient of 0.999. The mean percentage recovery was in the range of 97-98% which confirmed the accuracy of the method. The relative standard deviation of CS for precision was less than 2% which indicated that method was precise. The LOD and LOQ of CS were found to be 0.277 and 0.84 µg/ml, respectively. The percentage entrapment efficiency (%EE) and (%DL) of CS in formulation (F_5) was found to be 90±0.16 and 7.5±0.15%, respectively. Therefore, the method was effectively used for the estimation of CS in the bulk and also in nanostructured lipid carriers (NLCs).

Key words: CS, accuracy, central composite design, sample interval, nanostructured lipid carriers

INTRODUCTION

CS is chemically 5,7 dihydroxy flavone (Fig.1), which is ubiquitous in nature. It is obtained from honey, propolis, bitter melon, Himalayan pear, *Diaphragma juglandis fructus*, Walnut pellicle, flowers of common walnut, peel of passion fruit, Endophytic fungus (Chaetomium globosum), green marine algae, *Hyphaene thebaica, Chaetomium globosum* and *Cytisus villosus,* etc. (Stompor-Goracy *et al.*, 2021). It exhibits many pharmacological actions against cancer, diabetes, oxidation, inflammation, allergic reactions, hepatic diseases, neurological disorders, reproductive disorders and cardiovascular diseases (Naz *et al.*, 2019). Although several sophisticated methods such as ultra performance liquid chromatography tandem mass spectroscopy method (UPLC/LC-MS) (Ge *et al.*, 2015) and Ultra-high-performance liquid chromatography hybrid quadrapole time of flight mass spectroscopy (UHPLC-q-TOS-MS) (Koulis *et al.*, 2022) were developed for quantification of CS. Those were not economical, time consuming, and required more amount of mobile phase. In that scenario a simple, accurate, precise, cost effective, rapid and sensitive UV-visible spectrophotometer is the method of choice for estimation of CS in dosage forms. As per literature survey, no such method was available to quantify the CS in bulk and NLCs. In the present research study, analytical quality by design (AQbD) was utilized for the development of the novel UV-spectrophotometer method in quantification of CS in lipid nanoparticles. AQbD is the novel, simple tool for optimization of UV-visible instrumental operations (Scanning speed, scanning rate, slit width and wavelength) affecting analysis of analyte, etc. Along with it, aids in the risk assessment of the critical factor which are directly influenced in the presentation of the analytical method (Rohman *et al.*, 2018). In current study, CCD was chosen for optimizing UV-visible spectrophotometer method. For that

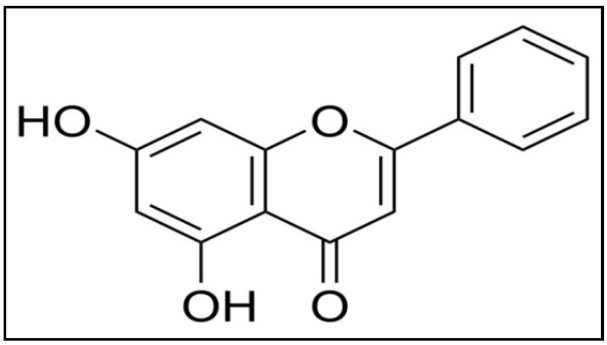

Fig. 1. Structure of *chrysin*.

instrumental variables such as scanning speed and sample interval were selected as independent factors. The main objective of the research was to reduce the variability during the measurement of drug by concept of AQbD. Therefore, to develop and validate the UV-visible spectrophotometer international conference of harmonization (ICH) guidelines Q2 (R1) were followed.

MATERIALS AND METHODS

CS was procured from the BLD Pharma Tech Pvt. Ltd., India. Methanol analytical grade and Tween 20 were purchased from Loba Chemie India, respectively. Double distilled water was used throughout the study. Capryol 90, Emulcire 61WL 2659 and Transcutol HP were gift samples from Gattefosse India. UV-visible spectrophotometer (1800-Shimadzu), Sample holder (1 cm capacity quartz cuvettes) and Analytical balance (Shimadzu) were used.

The selection of the solvent was the important step for the development of the method. For that based upon the literature survey, some of the solvents were selected to determine the solubility study of CS. The solubility studies of the CS were carried out in various suitable, selected solvents such as methanol, ethanol, DMSO, water and NaOH. Among them, CS showed highest solubility in methanol and transparent solution was formed (Dong *et al.*, 2021; Bansal *et al.*, 2022).

As per the ICH Q8 (R2) guidelines, AQbD was used for the optimization of UV-visible spectrophotometer. The impulsion for employing the AQbd approach for optimization of quality features of UV-visible spectrophotometer were for the achievement of appropriate experimental data. AQbd principle strategy helped in the establishment of the analytical target profile which could aid in the estimation of drug in samples. In that scenario to meet the analytical profile, CS absorbance was selected as critical analytical attribute (CAA). Furthermore, CCD was adopted as appropriate quality by design for evaluation and optimization of UV-visible spectrophotometer method. It was performed by varying the various parameters like sample interval and scanning speed (Shraddha Parmar, 2021). The above mentioned parameters were chosen as critical method variables (CMV) based upon the control noise experiments such as wavelength detection, sampling interval, scanning speed, sample integrity and variation of solvent. (Rohman *et al.*, 2018). To determine the maximum wavelength of the CS, a 10 µg/ml concentration solution of CS was scanned from 400-200 nm by taking methanol as a blank.

CS was detected at the wavelength of 268 nm. 2, 4, 6, 8 and 10 µg/ml of standard solutions were prepared in methanol and analyzed. As per the ICH Q2 (R1) guidelines, the developed method was validated. For the identification of the optimum conditions of the analytical method CCD was used. Based on the control noise experiment, sample speed and sample interval were selected as the CMV, which were further optimized by CCD. For that the response variable (absorbance) of CS was measured at 268 nm using 10 mg/ml standard solution.

To get the 100 µg/ml concentration of the standard stock solution, 10 mg of CS was dissolved in 100 ml of methanol. Further dilutions were carried out from 0.1 mg/ml concentration solution to get 2, 4, 6, 8 and 10 µg/ml solutions in to 10 ml volumetric flask and finally the volume made with methanol up to the 10 ml mark.

The analytical method validation was carried out, by evaluating linearity, accuracy, repeatability, specificity, limit of detection (LOD), limit of quantification (LOQ) and robustness according to the ICH guidelines Q2 (R1). The linearity curve of CS was constructed between the mean absorbance of five replicates verses corresponding concentration of CS. Then regression equation was calculated.

The accuracy of the developed method was evaluated on the basis of the absolute recovery of CS at three levels (80, 100 and 120%) of 6 µg/ml concentration sample. For that level, 80% of the sample was labelled as

LQC, level 100% of sample was labelled as MQC and 120% was labelled as HQC, respectively. To prepare these dilutions aliquots of 0.48, 0.60 and 0.72 ml were withdrawn from 0.1 mg/ml of stock solution and finally the volume of each sample was made up to 10 ml with methanol in 10 ml volumetric flask. Then the final concentration was made of 4.8, 6.0 and 7.2 µg/ml, respectively. Each study was performed three times and mean was collected. The following equation was used for calculating the percentage absolute recovery. In addition to that % RSD of the each sample was estimated following (Panda *et al.*, 2018; Gurrala *et al.*, 2022) as:

$$\text{Absolute percentage recovery} = \frac{\text{Actual concentration recovered}}{\text{Theoretical concentration}} \times 100 \quad \text{...Eq. 1}$$

Precision refers to the, measurement of the degree of variability among the data, which are obtained from a series of experiments conducted under similar conditions of uniform samples (Jolly, 2019). To check the precision of developed method, it was analyzed in the form of repeatability and intermediate precision. Repeatability was measured by taking the five absorbance values of different concentration samples such as LQC, MQC and HQC on the same day (intraday repeatability). To carry out intermediate (inter day) precision five absorbance values of LQC, MQC and HQC samples were measured on three different consecutive days along with three different analyst (inter analyst). The mean results of all samples (LQC, MQC and HQC) were recorded and % RSD was calculated.

LOD is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. It is calculated from standard deviation of response (σ) and slope of calibration curve (S) as per following formula (Vijayalakshmi, 2017).

$$\text{LOD} = \frac{3.3\,\sigma}{S} \quad \text{...Eq. 2}$$

LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy. It is calculated by formula (Vijayalakshmi, 2017).

$$\text{LOQ} = \frac{10\,\sigma}{S} \quad \text{...Eq. 3}$$

Where, σ is the standard deviation and S is the slope.

CS loaded NLCs were prepared by modified hot homogenization method followed by probe sonication technique. To prepare 200 ml of NLCs, 10 mg of CS was weighed and dissolved in Capryol 90 (40 mg) and added to the melted Emulcire 61 WL 2659 (200 mg) and heated above 5°C of melting point of lipids. Simultaneously, in a similar manner, Tween 20 and Transcutol HP (450 mg : 450 mg) was heated separately. This was followed by addition of lipid phase to the aqueous phase with stirring at 700 rpm on magnetic stirrer. Immediately, formed nano-emulsion was added to the hot water (200 ml) and the sample was homogenized for 45 min, then probe sonicated up to 5 min. Later the sample was kept aside for the recrystallization of lipids to obtain NLCs (Sharma *et al.*, 2021).

The % EE refers to the amount of drug incorporated in the dispersion system relative to the total drug added. It identified the proportion of the drug contained within the particles and the proportion of free drug remaining in the dispersion medium. To calculate it, A 1 ml aliquots of CS NLCs dispersion was centrifuged up to 15 min at 10000 rpm. Then the supernatant was collected and diluted with methanol. The sample analysis was followed by spectrophotometer at 268 nm (Madane and Mahajan, 2016). % DL refers to the percentage of drug loaded into the lipids corresponding to the total weight of the lipid added to the dispersion system. It was calculated by using centrifugation method and similar procedure followed as that of % EE calculation (Madane and Mahajan, 2016).

The % EE and DL of CS loaded NLCs were calculated using the following equations:

$$\%\ \text{EE} = \frac{\text{Amount of drug added} - \text{Amount of drug in supernatant}}{\text{Amount of drug added}} \times 100 \quad \text{...Eq (4)}$$

$$\%\ \text{DL} = \frac{\text{Amount of drug added} - \text{Amount of drug in supernatant}}{\text{Amount of drug added} + \text{Amount of lipid added}} \times 100 \quad \text{...Eq (5)}$$

RESULTS AND DISCUSSION

The highest solubility of the CS was observed in the methanol. As per the literature review CS is freely soluble in the methanol as well stable**.** The wavelength maxima of CS were determined from spectrum. It was found to be 268 nm, as shown in Fig. 2, and used for further analysis.

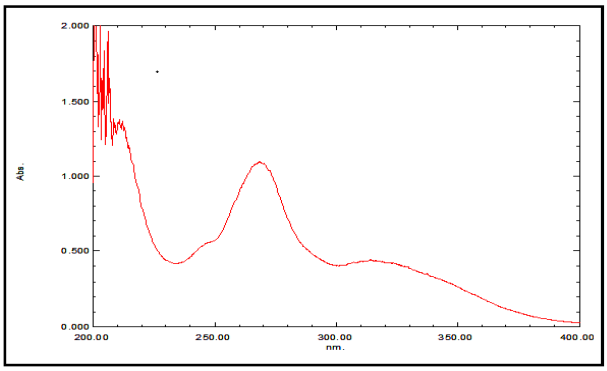

Fig. 2. The wavelength maxima of CS.

A total of 13 experiments were carried out based on CCD design to find out the influence of CMV on the CAA (Table 1). The CCD was given the optimized spectrophotometric conditions with scanning speed -0.600995 nm/ sec and sampling interval as -0.5176626 nm with desirability nearly 1 (Table 2). Fit summary of the model suggested the quadratic model as the best fit for CAA. Analysis of variance (ANOVA) generated the p-value less than 0.05, which indicted that the model was adequate. The polynomial equations generated by CCD had shown that scanning speed and sampling interval together had the significant negative impact on absorbance. However, there was positive effect individually on absorbance. Fit statistic was performed which represented the R^2 with 0.7687, indicating that the model

Table 1. The detailed information of 13 runs along with levels

Run No.	A	B
1	0	0
2	0	0
3	0	0
4	-1	-1
5	-1	0
6	1	1
7	1	-1
8	0	0
9	0	0
10	0	+1
11	-1	1
12	+1	0
13	0	-1

Table 2. Scanning speed and sampling interval

Level	Scanning speed	Sampling interval (nm)
-1 (Low)	Slow	0.5
0 (Medium)	Mediun	1.0
+1 (High)	Fast	2.0

was significant. The detailed information of ANOVA for quadratic model, lack of fit for scanning speed and sampling interval, data of model summary statistics, fit summary and fit statistics on absorbance is shown in Table 3. In addition numerical and graphical optimization is showing the range within the given design space. Furthermore, the effect of CMVs on CAA was studied by using response curve, contour and 3D plots using design expert software which are shown in Fig. 3. The contour plot represented the low level of scanning speeds and increasing sample interval as small increase in responsiveness. Similarly, at low levels of sample interval a curvilinear rise in responsive was seen. However, at low levels of both CMVs, a minimal absorbance was seen. The graphical optimization was performed with optimum CMV values. The actual vs. predicted plot illustrated that the data obtained from the experiments lie within the limit (Fig. 4). The obtained polynomial equation for model was as follows (Vancha *et al.*, 2022):

$$\text{Absorbance: } +0.776800+0.010110 \times A+0.012744 \times B-0.005250 \times A \times B+ 0.007850 \times A - 0.20900\, B \quad \text{...Eq. (6)}$$

Where, A is the scanning speed and B is the sampling interval.

The calibration curve of CS was plotted by taking the concentration on x-axis (µg/ml) and absorbance values on y-axis. A linear graph was obtained from 2-10 µg/ml range concentration with a correlation coefficient (R^2) value of 0.999 for CS (Fig. 5).

The accuracy of the developed method was evaluated on the basis of the absolute recovery of CS at three levels (80, 100 and 120%) of 6 µg/ml concentration sample. Then actual mean recovery concentrations of all three levels were calculated in µg/ml. The percentage recovery was found to be above the 95% and below 105%. The % RSD was used for the verification of the developed method accuracy, which was found to be below the < 2% (Table 4).

Table 3. The detailed information of ANOVA for quadratic model, lack of fit for scanning speed and sampling interval, data of model summery, fit summery and fit statistics on absorbance

Source	Sum of squares	d. f.	Mean squares	F-value	p-value
Model	0.0061	5	0.0012	4.65	0.0343 (Significant)
Scanning speed (A)	0.0008	1	0.0008	3.14	0.1197
Sampling interval (B)	0.0013	1	0.0013	4.99	0.0606
AB	0.0001	1	0.0001	0.42	0.5360
A2	0.0004	1	0.0004	1.65	0.2403
B2	0.0030	1	0.0030	11.67	0.0112
Residual	0.0018	7	0.0003		
Lack of fit	0.0008	3	0.0003	0.95	0.4938 (Not significant)
Pure error	0.0011	4	0.0003		
Cor total	0.0079	12			
Sequential model sum of squares					
Means vs total	7.68	1	7.68		
Linear vs. mean	0.0021	2	0.0011	1.84	0.2092
$2F_I$ vs. linear	0.0001	1	0.0001	0.1755	0.6851
Quadratic vs. $2F_I$	0.0038	2	0.0019	7.35	0.0190 (suggested)
Cubic vs. quadratic	0.0007	2	0.0003	7.35	0.3223
Residual	0.0012	5	0.0002	1.43	
Total	7.69	13	0.5916		
Lack of fit tests					
Linear	0.0047	6	0.0008	2.96	0.1568
2 F_I	0.0046	5	0.0009	3.46	0.1262
Quadratic	0.0008	3	0.0003	0.9577	0.4938 (suggested)
Cubic	0.0001	1	0.0001	0.3695	0.5761
Pure error	0.0011	4	0.0003		
Model summary statistics					
Source	Standard deviation	R^2	Adjusted R^2	Predicted R^2	Press
Linear	0.0240	0.2687	0.12224	-0.4329	0.0113
2 F_I	0.0251	0.2826	0.0435	-0.6689	0.0132
Quadratic	0.0161	0.7687	0.6035	0.1021	0.0071
Cubic	0.0152	0.8529	0.6471	-0.0062	0.0079
Fit statistics					
Standard deviation	0.0161	R^2	0.7687		
Mean	0.7688	Adjusted R^2	0.6035		
C.V. (%)	2.10	Predicted R^2	0.1021		
		Adeq Precision	8.1933		

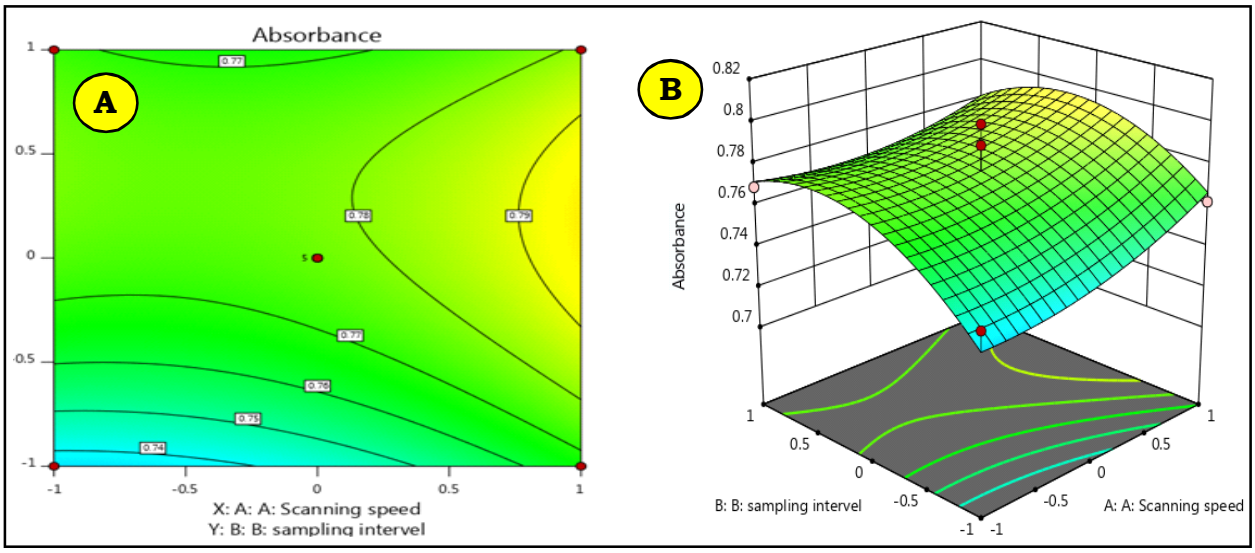

Fig. 3. The counter (A) and 3D plot (B) representing the effect of CMVs (scanning speed and sampling interval) on absorbance.

To check the precision of the developed method % RSD was used. It was measured for five absorbance values of the LQC, MQC and HQC solutions at intraday, inter day and inter analyst level by the identical experimental conditions. The obtained results indicated that

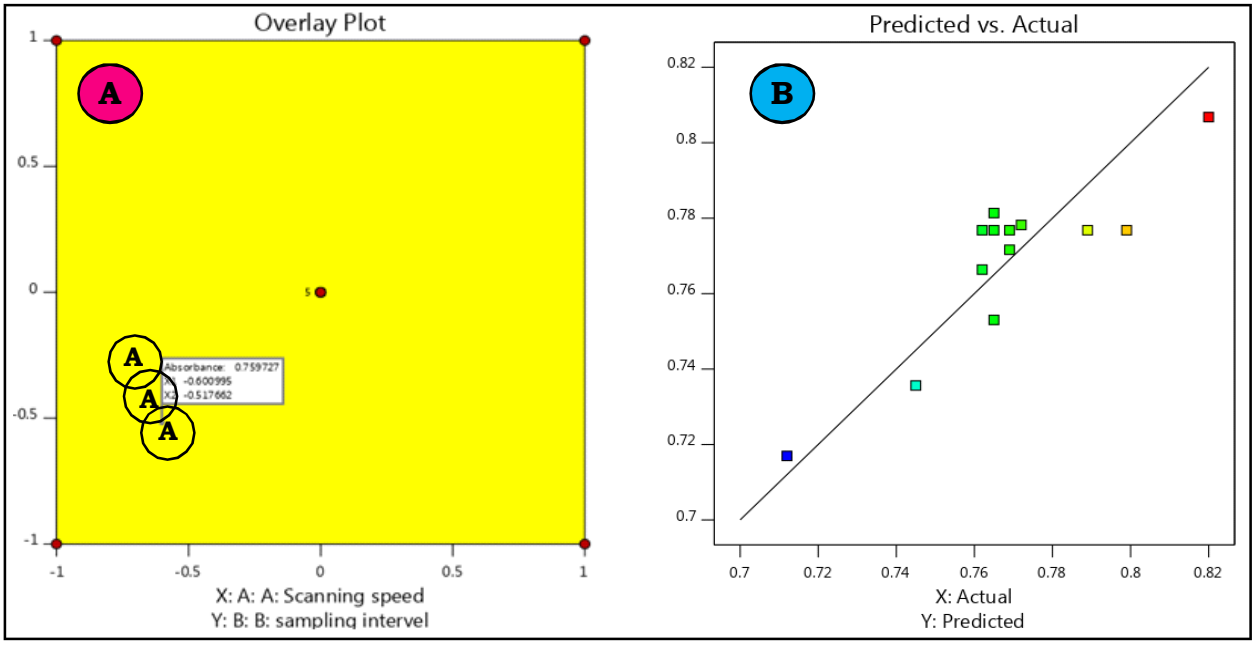

Fig 4. Over lay plot representing the optimized CMVs (A) and an illustration representing the correlation between the actual and predicted values (B).

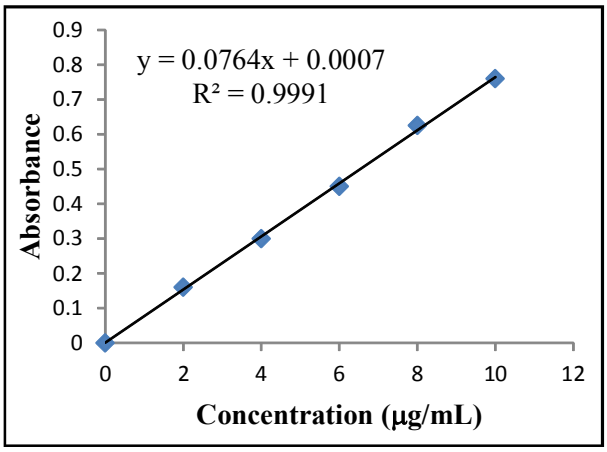

Fig. 5. Calibration curve of CS.

Table 4. Accuracy results

Levels	Concentration of standard solution (µg/ml)	Acutal mean recovery concentration (µg/ml)	% Recovery
LQC	4.8	4.73±0.24	97.91±0.26
MQC	6.0	5.92±0.25	98.60±0.25
HQC	7.2	7.11±0.34	98.86±0.32

the developed method was suitable and précised. It was confirmed by the results of %RSD i.e. below the 2% (Table 5).

The LOD and LOQ for the developed UV method of CS in methanol was found to be 0.277 and 0.84 µg/ml, respectively.

The developed formulations were analyzed for the % EE and DL in the NLCs by using the equations 4 and 5 (Table 6).

Table 5. Precision results

Parameters intraday	Levels	Conc. (µg/ml)	Mean absorbance (n=5)	% RSD
Intraday				
Repeatability	LQC	4.8	0.3588±0.0071	1.94
	MQC	6.0	0.4546±0.0054	1.20
	HQC	7.2	0.6262±0.0087	1.40
Interday				
Day 1	LQC	4.8	0.3588±0.0071	1.99
	MQC	6.0	0.4546±0.0054	1.20
	HQC	7.2	0.6262±0.0087	1.40
Day 2	LQC	4.8	0.3598±0.0055	1.55
	MQC	6.0	0.4782±0.0085	1.78
	HQC	7.2	0.6237±0.0117	1.88
Day 3	LQC	4.8	0.354±0.0065	1.86
	MQC	6.0	0.4658±0.0088	1.90
	HQC	7.2	0.6252±0.0109	1.75
Inter analyst				
Analyst 1	LQC	4.8	0.3586±0.0069	1.94
	MQC	6.0	0.485±0.0089	1.84
	HQC	7.2	0.6262±0.00117	1.87
Analyst 2	LQC	4.8	0.3608±0.0068	1.89
	MQC	6.0	0.4602±0.0072	1.56
	HQC	7.2	0.635±0.0111	1.76
Analyst 3	LQC	4.8	0.3548±0.0065	1.84
	MQC	6.0	0.4662±0.0090	1.95
	HQC	7.2	0.626±0.0118	1.90

CONCLUSION

A novel simple, robust, cost effective AQbD based UV-spectrophotometric method was successfully developed and validated. Statistical analysis results of validation technique confirmed the established method suitable for application in quality control laboratory. The developed method was applied in the estimation of CS in NLCs effectively. All the obtained results of

Table 6. The % EE and DL results of NLCs

Formulation	F_1	F_2	F_3	F_4	F_5
% EE	67±0.12	75±0.22	78±0.23	89±0.06	90±0.06
% DL	6.23±1.13	8.5±0.36	7.5±0.24	8±0.06	7.5±0.15

validation parameters lied within the limits. The percentage recovery of CS lied within the limit and it was found to be 97.91 - 98.86% at 268 nm wavelength. The LOD and LOQ values of CS were found to be 0.277 and 0.84 µg/ml, respectively. The precision study results were found to be <2% RSD, which indicated that the method was within the acceptable range. Therefore, the developed method was accurate, reliable and precise, which can be used for the estimation of CS in the bulk along with nano formulations.

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