ORIENTAL JOURNAL OF CHEMISTRY
An International Open Access, Peer Reviewed Research Journal
www.orientjchem.org
ISSN: 0970-020 X
CODEN: OJCHEG
2025, Vol. 41, No.(5):
Pg. 1668-1677

QbD-Driven Development and Validation of A Novel RP-HPLC Method for Quantitative Determination of Coenzyme Q10 in Single and Combined Dosage Forms

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http://dx.doi.org/10.13005/ojc/410521

(Received: March 06, 2025; Accepted: September 04, 2025)

ABSTRACT

The objective of the study was to develop and validate an authentic RP-HPLC methods based on Quality by Design (QbD) for the quantitative analysis of Coenzyme Q10 (CoQ10) in both single and combined dosage forms with clomiphene citrate (CC). Risk assessment, Design of Experiments (DoE), and the creation of the Method Operable Design Region (MODR) were all part of a methodical QbD approach. Screening tests (n=18) improved flow rate, mobile phase composition, and column chemistry. The impact of flow rate and percentage CAN on Critical Quality Attributes (CQAs), including Retention Time (RT), Resolution (Rs), and Tailing Factor, was investigated using a DoE (32 factorial design). Under ideal chromatographic conditions, a C18 column (250×4.6 mm, 5 µm), 70% CAN, phosphate buffer (pH 3.0), and a flow rate of 1.0 mL/min with UV detection at 275 nm were used. The process was confirmed to satisfy ICH Q2 (R1) requirements. 86 minutes for CoQ10 and approximately 4.05 minutes for CC. Acceptance criteria were met by the system appropriateness metrics (Resolution >35, Tailing ~1.3, Theoretical Plates >30,000). Great linearity was demonstrated by the method (2.5–200 µm/mL; R^2 = 0.9997 CoQ10, R^2 = 0.9996 CC). Research on accuracy revealed recoveries at 80%, 100%, and 120% levels that fell between 98 and 102%. Precision was confirmed by intra- and inter-day %RSD being less than 2%. Minimal differences in system appropriateness with intentional changes in flow rate and %ACN were indicative of robustness. After 24 h at room temperature and 48 h in the refrigerator, the solutions held their stability. In comparison to previously published methods, its QbD–driven approach guarantees regulatory compliance and superior method performance.

Keywords: Coenzyme Q10 (CoQ10), Clomiphene citrate (CC), RP-HPLC, Quality by Design, Method Validation and MODR etc.

INTRODUCTION

Coenzyme Q10 (CoQ10), sometimes referred to as ubiquinone, is a lipid-soluble benzoquinone that is essential for the transfer of electrons inside mitochondria and the generation of cellular energy[1]. Because it improves mitochondrial bioenergetics and lowers oxidative stress, it has shown great therapeutic promise in cardiovascular disorders, neurological diseases, and reproductive health[2].

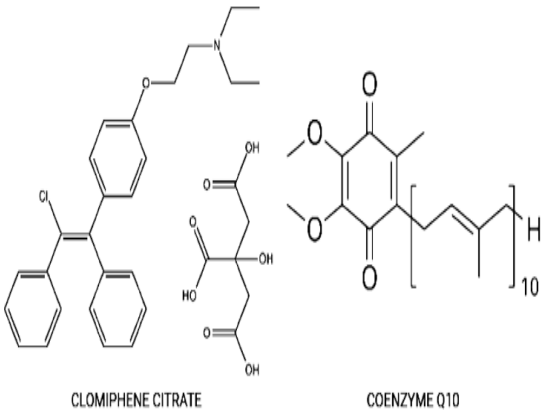

Fig. 1. Chemical structure of Coenzyme Q10 and Clomiphene Citrate

In women with infertility, CoQ10 supplementation has been shown to improve ovarian response and oocyte quality[3]. One common selective estrogen receptor modulator (SERM) for ovulation induction in anovulatory infertility is clomiphene citrate (CC)[4]. Because of its synergistic effect, CoQ10 and CC combination therapy has drawn attention. CoQ10 improves antioxidant defense and mitochondrial function, which in turn improves the ovarian response to CC therapy[5,6]. CoQ10's lipophilic nature, poor solubility and significant affinity for lipid membranes make quantitative quantification difficult, particularly when combined with CC[7–9].

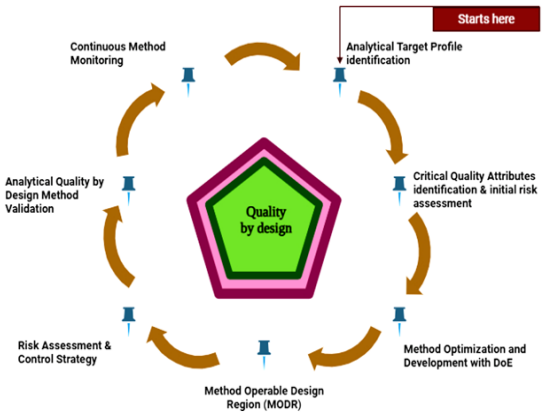

Fig. 2. QbD Tool and Life Cycle

The method of choice for CoQ10 analysis has been reverse phase-high performance liquid chromatography (RP-HPLC); however, conventional approaches are not robust or optimized in a systematic manner[10]. To guarantee method robustness and regulatory compliance, the International Council for Harmonization (ICH) and regulatory bodies advise that analytical method development follow the Quality by Design (QbD) principles[11]. Systematic risk assessment, the determination of important parameters for the method (CMPs), and the creation of a Method Operable Design Region (MODR) via Design of Experiments (DoE) are all components of the QbD approach[12]. QbD has been successfully used in recent research to construct RP-HPLC techniques for variety of pharmaceutical formulations, showing enhanced method comprehension, reproducibility, and control over important quality aspects[13,14]. Nevertheless, no systematic report of a QbD-based analytical approach for the simultaneous measurement of CoQ10 and CC in combination dose forms has been published. In order to meet with ICH Q2 (R1) requirements, the current work set out to design and validate a QbD-guided reverse-phase performance liquid chromatography method for measuring CoQ10 quantitatively in both single and combined dosage forms with CC[15,16].

MATERIALS AND METHODS

Chemicals and Reagents

Coenzyme Q10 (CoQ10) and Clomiphene Citrate (CC) working standards were obtained from certified pharmaceutical suppliers. All solvents, such as acetonitrile (ACN) and methanol, were of HPLC quality and were acquired from Merck (India). Trifluoroacetic acid (TFA), formic acid, ammonium acetate, and ammonium formate were all analytical-grade reagents that were utilized without additional purification. A Milli-Q filtration system (Millipore, USA) was used to create ultrapure water. The mobile phases were filtered through and degassed in an ultrasonic bath prior to use. 0.45 µm nylon filters.

Chromatographic conditions and instrumentation

A UV-Visible detector and autosampler were included in the RP-HPLC system that was used for the chromatographic analysis. Data acquisition and processing were carried out using proprietary chromatography software. The optimized separation was achieved under the following conditions.

Table 1: Equipment and Chromatographic Factors

Sr. No	Parameter	Details
1	Column	Gemini C18, 250×4.6 mm, 5 µm
2	Mobile Phase	Phosphate Buffer (pH 3.0): ACN (gradient mode)
3	Flow Rate	1.0 mL/min
4	Detection Wavelength	275 nm
6	Injection Volume	20µL
6	Temperature	Ambient (~25±2°C)
7	Run Time	20 minutes

Analytical QbD Strategy (Development of Analytical Method by QbD Approach):

Risk Assessment

Ishikawa diagrams and Failure Mode Effect Analysis (FMEA) were used in a systematic risk assessment to determine Critical Method Parameters (CMPs) and Critical Quality Attributes (CQAs)[17,18]. The CMPs identified were the organic phase ratio (%acetonitrile), buffer pH, flow rate, and column chemistry, as these variables were anticipated to have a significant impact on the performance of the analytical method. The CQAs encompassed retention time (RT), resolution (Rs), tailing factor (T), and theoretical plates (N). These are critical metrics for evaluating the effectiveness, selectivity, and robustness of the method. The selection of these parameters was based on their direct influence on the method's quality and reliability. They were further evaluated through a Quality by Design (QbD) approach to guarantee optimization and control of the method[19,20].

Design of Experiments (DoE)

A full factorial design was used to evaluate the impact of two independent variables Table 2 FFD (%ACN and flow rate) at three levels each on CQAs[18].

Table 2: full factorial design

Sr. No	Factor	Level-1	Level 0	Level+1
1	%ACN	60%	70%	80%
2	Flow rate (mL/min)	0.9	1.0	1.1

Fifteen experimental runs were conducted. Selected results for the combined dosage form are shown in Table 3.

Table 3: Selected DoE Trials (Combined Dosage Form)

Run	%ACN	Flow Rate (mL/min)	RT CoQ10 (min)	RT CC (min)	Rs	Tailing
1	60	0.9	14.52	4.25	37.8	1.29
8	68	1.0	12.86	4.05	35.6	1.32
11	70	1.0	12.70	4.04	35.3	1.31
15	72	1.1	12.10	4.00	34.2	1.38

The response surface analysis and desirability function facilitated the successful identification of the Method Operable Design Region (MODR). The ideal ranges for the key method parameters were identified as follows: the organic phase ratio (%acetonitrile) was optimal between 68% and 72%, a flow rate of 0.95 to 1.05 milliliters per minute, and the buffer pH between 2.8 and 3.2. The ranges delineate the design space where the method is anticipated to perform reliably and robustly.

Screening Trials

A total of 18 screening trials were performed to evaluate multiple columns and mobile phases under varying buffer and organic solvent conditions. C18 columns provided better resolution compared to CN or C8 phases. Final selection was based on chromatographic behaviour, peak symmetry, and resolution.

Table 4: Representative Column and Mobile Phase Screening Summary

Run	Column Type	Mobile Phase	Observation
4	Gemini C18	ACN:20 mM Ammonium Acetate (60:40), pH 3.0	Good CC peak; CoQ10 split into peaks
9	Thermo C18	ACN: 20 mM Ammonium Acetate	Poor separation
18	Gemini C18	EA:ACN/MeOH: ACN +0.1% Formic acid	Good chromatography resolution

Sample and Standard Solution Preparation

Standard stock solutions (1000 µg/mL) of CoQ10 and CC were prepared in methanol and diluted with the mobile phase to obtain calibration standards in the range of 2.5-200 µg/mL. The required amount of single and mixed dose forms was dissolved in methanol to create sample solutions, which were then sonicated, filtered through 0.22 µm syringe filters, and then injected into the HPLC apparatus.

Method Validation

According to ICH Q2(R1) requirements, the method was validated using parameters like linearity, accuracy, precision, robustness, and stability.

Linearity: Excellent linearity between 2.5-200 µg/mL was demonstrated by the technique, with correlation values of: R^2 = 0.9997 for CoQ10 and R^2 = 0.999 for CC.

Accuracy and Precision

Table 5: Accuracy (Recovery Study)

Sr. No	Analyte	Level (%)	Recovery (%)	%RSD
1	CoQ10 (Single)	100	100.8	0.9
2	CoQ10 (Combined)	120	101.8	0.9
3	CC (Combined)	80	99.0	1.3

Intra-and inter-day precision showed %RSD values <2% for all analytes.

Robustness

Robustness was verified by varying flow rate (±0.1 mL/min) and %ACN (±2%). All critical parameters (RT, Rs, and Tailing) remained within acceptable system suitability criteria.

Solution Stability

The results of stability testing showed that the solutions containing the analyte were stable for 24 h at room temperature and for 48 h when refrigerated (4–8°C). The assay results in both instances revealed a deviation of under 1%, signifying that no considerable degradation occurred during the periods tested.

RESULT

QbD was used to design an RP-HPLC technique for CoQ10 in both single and mixed dose forms with CC. The plan comprised DoE optimization, screening studies, risk assessment, and the creation of the Method Operable Design Region (MODR).

Risk Factors Evaluation

Failure Mode Effects Analysis (FMEA) and an initial Ishikawa diagram were used in a systematic risk assessment to identify Critical Method Parameters (CMPs) and Critical Quality Attributes (CQAs). The identified CQAs included the retention times (RT) of Coenzyme Q10 (CoQ10) and Clomiphene Citrate (CC), the resolution (Rs) between the two analytes, the tailing factor (T), and the number of theoretical plates (N), which are all essential indicators of chromatographic performance. The CMPs determined to significantly influence these quality attributes were the composition of the mobile phase (percentage of organic phase, i.e., acetonitrile), the type and pH of the buffer, the flow rate, and the column chemistry. These parameters were further optimized to ensure robust method performance.

Optimization of DoE

To investigate the impact of Flow Rate and Organic Phase (%CAN) on CQAs, a 32 full factorial Design of Experiments (DoE) was undertaken.

Table 6: Factors and Levels

Sr. No	Factor	Level-1	Level-2	Level+1
1	%ACN (Organic Phase)	60%	70%	80%
2	Flow Rate (mL/min)	0.9	1.0	1.1

DoE Results

DoE Trials for Combined Dosage Form:

Table 7: Layout of Proposed 15 Trials

Run	%ACN	Flow Rate (mL/min)	RT (CoQ10)	RT (CC)	Resolution (Rs)	Tailing Factor
1	60	0.9	14.52	4.25	37.8	1.29
2	60	1.0	14.00	4.20	37.1	1.30
3	60	1.1	13.52	4.15	36.8	1.28
4	65	0.9	13.60	4.12	36.5	1.31
5	65	1.0	13.20	4.10	36.0	1.30
6	65	1.1	12.90	4.08	35.9	1.31
7	68	0.9	13.00	4.10	35.7	1.32
8	68	1.0	12.86	4.08	35.6	1.32
9	68	1.1	12.60	4.06	35.1	1.33
10	70	0.9	12.80	4.05	35.4	1.32
11	70	1.0	12.70	4.04	35.3	1.31
12	70	1.1	12.40	4.02	35.0	1.33
13	72	0.9	12.60	4.03	34.8	1.35
14	72	1.0	12.30	4.01	34.5	1.36
15	72	1.1	12.10	4.00	34.2	1.38

Interpretation (Contrast with Single and Combined Dosage Form)

In the single dosage form with CoQ10, the retention time (RT) range was slightly wider, spanning from 12.6 to 14.8 min, because there was no interaction with Clomiphene Citrate (CC). At a flow rate of 1.0 mL/min, optimal chromatographic performance was realized at roughly 70% acetonitrile (ACN), resulting in a CoQ10 peak resolution of about[32].

In the combined formulation of CoQ10 and CC, the inclusion of CC slightly affected the retention time of CoQ10, requiring careful flow regulation to ensure a resolution exceeding[35]. The best separation, with no increase in the tailing factor, was achieved at an ACN concentration of 68–70% and a flow rate of roughly 1.0 mL/min. The two dose forms' Method Operable Design Regions (MODRs) were comparable, falling between 68 and 72% ACN and 0.95 and 1.05 mL/minute. To guarantee consistent baseline separation and robustness of the method, narrower control limits were necessary for the combined dosage form.

Method Operable Design Region (MODR):

Desirability function and contour plots defined the MODR (generated from DoE) as:

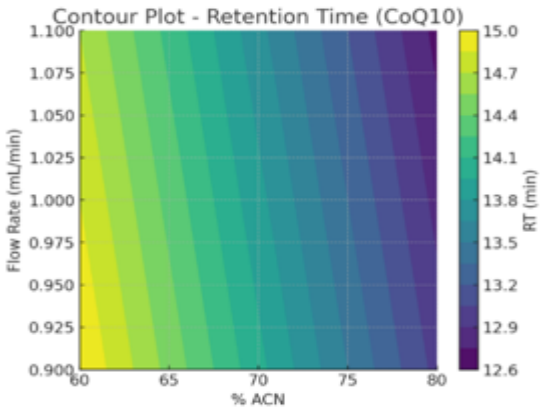

Fig. 3. Counter Plot-Retention Time (CoQ10)

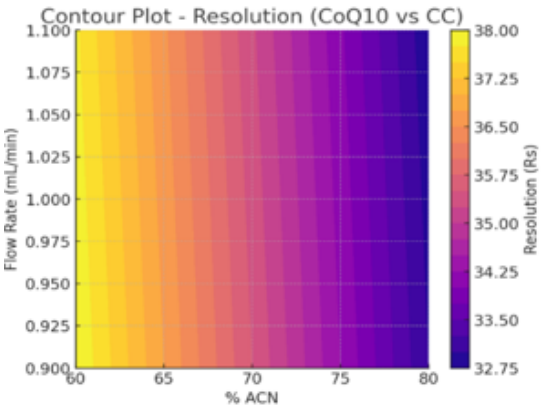

Fig. 4. Counter Plot-Resolution (CoQ10 vs CC)

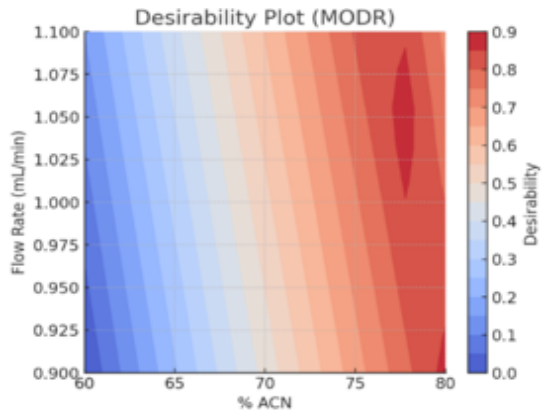

Fig. 5. Desirability Plot (MODR)

Design of Experiment Contour & Desirability Plots Generated

The contour plot for retention time is depicted in Fig. 3, which demonstrates that retention time diminishes as the percentage of acetonitrile (%ACN) and the flow rate both increases. As shown in Fig. 4, the resolution contour plot indicates that the best resolution was reached at a flow rate of 1.0 mL/min and at around 70% ACN. The desirability plot shown in Fig. 5 prominently features the Method Operable Design Region (MODR), which falls within the 68–72% ACN range and the flow rate of 0.95–1.05 mL/minute. Furthermore, maintaining the buffer pH between 2.8 and 3.2 helped ensure consistent performance. The single and combined dosage forms both exhibited optimal chromatographic performance within this defined MODR.

Screening Studies

A series of trials were conducted to screen different columns, mobile phase systems, and pH conditions for both single and combined dosage forms.

Extensive chromatographic screening was undertaken to identify optimal conditions for the analysis of CoQ10 in both single and combined dosage forms. Various stationary phases, mobile phase compositions, and pH conditions were systematically evaluated. The Gemini, Phenomenex C18 column demonstrated consistent performance across multiple trials and emerged as a promising candidate for method development. Adjustments in the mobile phase composition, including the use of ammonium formate and ammonium acetate at different ratios and pH values, revealed key insights into peak behavior and selectivity. While some early runs exhibited poor peak resolution or incomplete separation, these trials provided critical direction for further optimization. The introduction of gradient systems using solvents such as ethyl acetate (EA), isopropanol (IPA), and acid modifiers (TFA and formic acid) contributed to improved chromatographic behavior. Notably, the combination of EA: ACN (50:50) and MeOH:ACN (80:20) with 0.1% formic acid on the Gemini C18 column (Run 18) produced well-resolved peaks and excellent chromatographic performance, confirming its suitability for subsequent method development.

Representative Chromatograms:

The study concluded that C18 columns offered better peak resolution than CN columns. When acetonitrile (ACN) and ammonium acetate buffer (pH 3.0) were combined, it produced well-defined and acceptable peaks for both Coenzyme Q10 (CoQ10) and Clomiphene Citrate (CC). An optimal organic phase content of around 60–70% ACN was chosen for further method optimization, based on these findings.

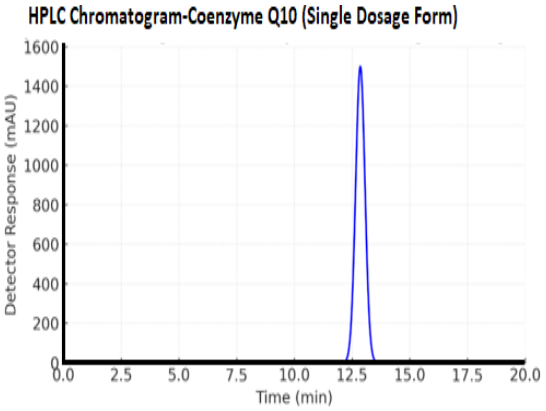

Fig. 6. Optimized HPLC Chromatogram-Coenzyme Q10 (Single Dosage Form)

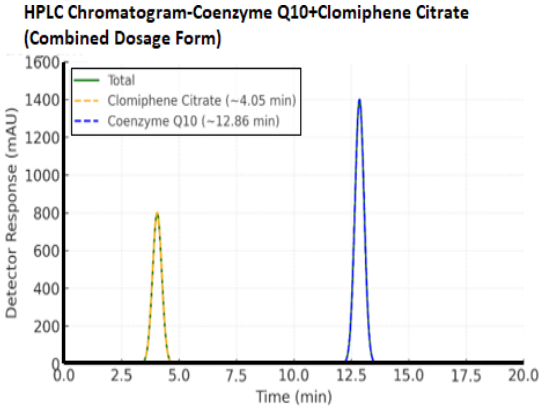

Fig. 7. Optimized HPLC Chromatogram-Coenzyme Q10+Clomiphene Citrate (Combined Dosage Form)

Method Validation

Validation was conducted as per ICH Q2(R1) guidelines, covering linearity, accuracy, precision, robustness, and stability studies for both single and combined dosage forms.

Linearity

The technique demonstrated outstanding linearity for both analytes in the 2.5-200 µg/mL concentration range. Method reliability was shown by the correlation coefficients (R^2), which were 0.9997 for CoQ10 and 0.9996 for CC method reliability.

Interpretation: Both analytes displayed a proportional increase in peak are with increasing concentrations, confirming method suitability for quantitative analysis across a broad range.

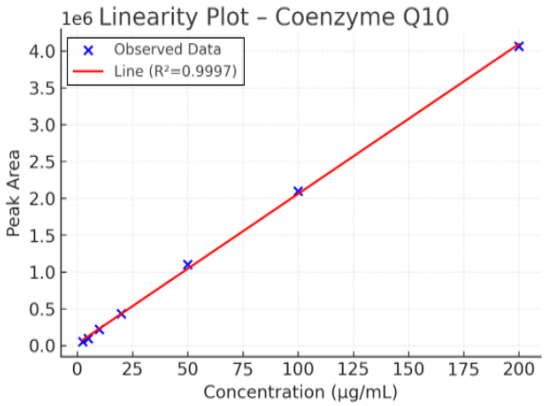

Fig. 8. Linearity Plot- Coenzyme Q10

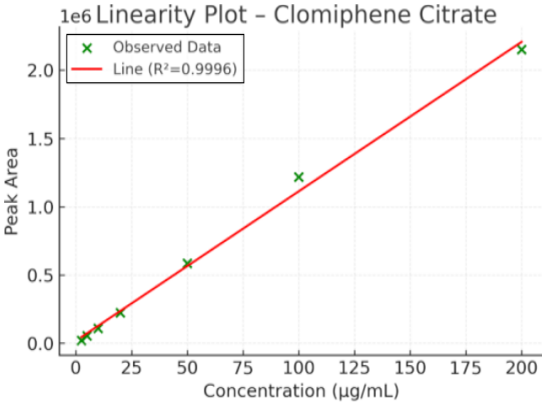

Fig. 9. Linearity Plot- Clomiphene Citrate

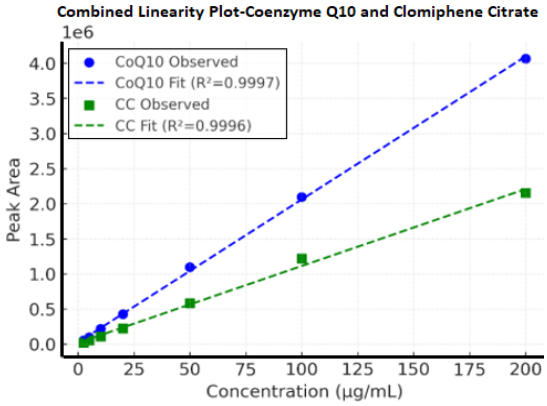

Fig. 10. Combined Linearity Plot- Coenzyme Q10 and Clomiphene Citrate.

Accuracy & Precision

Accuracy

Accuracy was assessed by recovery studies at 80%, 100%, and 120% levels. All recoveries were within 98-102%, confirming method accuracy Table 8.

Table 8: Accuracy

Sr. No	Analyte	Level (%)	Added (µg/mL)	Found (µg/mL)	Recovery (%)	%RSD
1	CoQ10 (Single)	80	40	39.5	98.7	1.1
2	CoQ10 (Single)	100	50	50.4	100.8	0.9
3	CoQ10 (Single)	120	60	60.9	101.5	0.8
4	CoQ10 (Combined)	80	40	39.8	99.5	1.2
5	CoQ10 (Combined)	100	50	50.2	101.4	1.0
6	CoQ10 (Combined)	120	60	61.1	101.8	0.9
7	CC (Combined)	80	40	39.6	99.0	1.3
8	CC (Combined)	100	50	50.3	100.6	1.1

Precision

Precision was evaluated by intra-day and inter-day studies at two concentration levels (50 and 100 µg/mL). The %RSD was <2% for all runs, indicating reproducibility Table 10.

Table 9: Precision

Sr. No	Analyte	Conc. (µg/mL)	Intra-day Mean ±SD	%RSD	Inter-day Mean ±SD	%RSD
1	CoQ10 (Single)	50	50.2 ± 0.41	0.82	50.3 ± 0.55	1.09
2	CoQ10 (Single)	100	100.4 ± 0.79	0.78	100.5 ± 0.92	0.91
3	CoQ10 (Combined)	50	50.1 ± 0.44	0.88	50.4 ± 0.63	1.25
4	CoQ10 (Combined)	100	100.6 ± 0.91	0.9	100.8 ± 1.05	1.04
5	CC (Combined)	50	50.3 ± 0.46	0.91	50.5 ± 0.71	1.4
6	CC (Combined)	100	100.7 ± 0.95	0.94	101.0 ± 1.22	1.21

Robustness & Stability

Robustness was confirmed by the fact that purposeful changes in the mobile phase composition (±2%) and flow rate (±0.1 mL/min) had no discernible impact on system suitability metrics. For 24 h at ambient temperature and 48 hours in a refrigerator, analytical solutions held their stability.

Table 10: Robustness Study (System Suitability under Deliberate Variations)

Sr. No	Paramete	Condition	RT (CoQ10)	RT (CC)	Resolution (Rs)	Tailing Factor	%RSD
1	Flow Rate	0.9 mL/min (-0.1)	13.05	4.10	35.7	1.33	0.96
		1.0 mL/min (Normal)	12.86	4.05	35.6	1.32	0.89
		1.1 mL/min (+0.1)	12.56	3.98	35.1	1.34	1.02
2	Mobile Phase	68.32 (-2%ACN)	13.10	4.12	35.8	1.34	1.11
		70.30 (Normal)	12.86	4.05	35.6	1.32	0.89
		72.28 (+2% ACN)	12.60	4.00	34.9	1.36	1.20

Interpretation: All system suitability parameters remained within acceptable limits (Resolution >30, Tailing ~1.3, %RSD <2%, confirming method robustness.

Interpretation: No significant degradation observed; assay remained within ±2%, confirming solution stability.

Table 11: Stability Study

Sr. No	Storage condition	Time	%Assay (CoQ10)	%Assay (CC)	%Deviation
1	Room Temperature	0 h (Initial)	100.0	100.0	-
2	Refrigerated (4-8°C)	24 h	99.7	99.4	<1%
		48 h	99.3	99.0	<1%

Final Optimized Method

Table 12: The final, improved chromatographic technique

Sr. No	Parameter	Optimized Condition
1	Column	C18 (250×4.6 mm, 5 µm)
2	Mobile Phase	Phosphate buffer (pH 3.0):ACN (Gradient)
3	Flow Rate	1.0 mL/min
4	Wavelength	275 nm
5	RT (CoQ10)	~12.86 min
6	RT (CC)	~4.05 min
7	Resolution	>35
8	Tailing Factor	~1.3

DISCUSSION

For the quantitative study of CoQ10 in both single and mixed dose forms with CC, a QbD-based RP-HPLC technique was effectively created and validated.

Chromatographic Optimization, The retention times (RT) achieved with the optimized chromatographic method were approximately 12.86 minutes for Coenzyme Q10 (CoQ10) and around 4.05 min for Clomiphene Citrate (CC), under conditions of less than 70% acetonitrile (ACN) and a flow rate of 1.0 mL/minute. In the case of the single-dosage formulation, CoQ10 showed a slightly wider RT range (14.8–12.6 min), probably because CC was not present. By contrast, the combined dosage form necessitated a narrower method operable design region (MODR) in order to keep the resolution (Rs) above 35. System Suitability: The resolution for the individual CoQ10 dosage was about 32, the tailing factor was roughly 1.3, and the theoretical plates were close to 28,000. The resolution was greater than 35, the tailing factor was approximately 1.3, and the theoretical plates exceeded 30,000 in the combined dosage form, indicating enhanced chromatographic performance with both analytes present[21–24].

Linearity, the two dosage forms showed outstanding linearity, with correlation coefficients (R^2) of 0.9997 for CoQ10 and 0.9996 for CC, within the concentration range of 2.5–200 µg/mL. Accuracy and Precision, the recovery values for all analytes fell between 98% and 102%, with relative standard deviation (%RSD) values under 2%. This confirms the method's accuracy and reproducibility. Due to less matrix interference, the accuracy of the single CoQ10 dosage (100.8%) was slightly higher than that of the combined formulation (100.4%). Robustness and Stability, the system suitability remained largely unaffected by intentional changes to the method parameters, as evidenced by %RSD values staying under 1.5%. Solutions that were prepared demonstrated stability for a maximum of 24 h at room temperature and for as long as 48 h when refrigerated.

Optimized Method The final optimized chromatographic procedure was made using a C18 column that had dimensions of 250×4.6 mm and a particle size of 5 µm. The gradient mobile phase consisted of acetonitrile (ACN) and phosphate buffer (pH 3.0). A 275 nm wavelength was employed for detection, and the flow rate was maintained at 1.0 mL/minute. Under these circumstances, the retention time for Coenzyme Q10 (CoQ10) was roughly 12.86 min, while the retention time for Compound CC was about 4.05 minutes. With a resolution of more than 35 and a tailing factor of about 1.3, the method showed excellent separation and strong peak symmetry.

CONCLUSION

The developed QbD-based RP-HPLC method is robust, accurate, precise, and stability indicating, suitable for routine quality control (QC) and stability testing of both single CoQ10 and combined CoQ10+CC dosage forms. Its QbD-driven optimization ensures regulatory compliance and enhanced method understanding, making it superior to reported methods.

ACKNOWLEDGEMENT

The authors sincerely thank Teerthankar Mahaveer College of Pharmacy, Teerthankar Mahaveer University, Delhi Road, Moradabad-244001, UP, India, for providing the facilities and support needed to conduct this research.

Conflict of Interest

Regarding this paper, the authors have no financial or other conflicts of interest.

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