Microchemical Journal 224 (2026) 117667

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Design of experiments-based development and validation of RP-HPLC and UV spectroscopic methods for the simultaneous determination of citicoline and nimodipine in pharmaceutical formulations

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ARTICLE INFO

Keywords:
UV
HPLC
Optimization
Design of experiment
Central composite design
Citicoline
Nimodipine

ABSTRACT

A Design of Experiments (DoE) approach was applied for the development and validation of RP-HPLC and UV spectrophotometric methods for the simultaneous determination of citicoline and nimodipine in a synthetic pharmaceutical dosage form. UV spectrophotometric analysis was performed using a simultaneous equation method in methanol, with detection at 239 nm for nimodipine and 271 nm for citicoline. RP-HPLC method development was carried out using a Central Composite Design to investigate the effects of buffer pH, flow rate, and organic solvent composition on critical chromatographic responses. Multi-response optimization was achieved using Derringer's desirability function, considering retention time, capacity factor, and resolution. The optimized chromatographic conditions consisted of methanol (40%) and phosphate buffer (60%) at pH 3.8 (adjusted with orthophosphoric acid), a flow rate of 1.0 mL/min, and UV detection at 225 nm using a C18 column. Citicoline and nimodipine were well resolved with retention times of 3.007 and 5.741 min, respectively. Both methods were validated in accordance with ICH Q2 (R1) guidelines. Statistical evaluation using ANOVA confirmed the significance of model terms and the reliability of the developed RP-HPLC method. The proposed methods are simple and reliable, with the DoE-guided approach providing improved method understanding and robustness, making them suitable for routine quality-control analysis of pharmaceutical formulations.

1. Introduction

Citicoline (Fig. 1a), also known as cytidine diphosphate choline (CDP-choline), is a key intermediate in the biosynthesis of phospholipids, particularly phosphatidylcholine, which is essential for neuronal membrane structure and function [1]. Phosphatidylcholine plays a vital role in maintaining cellular integrity within the central nervous system. Citicoline serves as a choline donor, where choline acts as a precursor for acetylcholine, a critical neurotransmitter involved in memory, learning, and cognitive processes. In addition to its neurotransmitter-related functions, citicoline supports neuronal repair and membrane stabilization, especially in ischemic and neurodegenerative conditions [2].

Nimodipine (Fig. 1b) is a dihydropyridine calcium channel blocker with high selectivity for cerebral vasculature and is primarily indicated for the management of subarachnoid hemorrhage (SAH) [3]. It inhibits calcium ion influx through voltage-dependent L-type calcium channels in vascular smooth muscle, leading to cerebral vasodilation and improved cerebral blood flow. This targeted pharmacological action is particularly effective in preventing delayed cerebral ischemia associated with SAH [4].

High-performance liquid chromatography (HPLC) is widely employed for pharmaceutical analysis due to its high precision, reproducibility, and capability to separate complex mixtures. Chromatographic separation efficiency depends on several factors, including mobile phase composition, buffer pH, column characteristics, and flow rate. Among these, mobile phase pH plays a critical role by influencing the ionization behavior of analytes, thereby affecting retention, selectivity, and resolution [5]. Careful optimization of these parameters is especially important when simultaneously analyzing structurally and physicochemically dissimilar compounds such as citicoline and nimodipine.

In recent years, analytical method development has increasingly

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https://doi.org/10.1016/j.microc.2026.117667
Received 17 December 2025; Received in revised form 24 February 2026; Accepted 10 March 2026
Available online 18 March 2026

adopted a risk-based and systematic framework based on Analytical Quality by Design (AQbD) principles to ensure robustness and regulatory compliance [6]. This approach integrates predefined analytical objectives, risk assessment, and multivariate optimization tools to identify and control critical method parameters (CMPs). Design of Experiments (DoE), particularly response surface methodologies such as Central Composite Design (CCD), enables quantitative evaluation of factor–response relationships and establishment of a reliable design space. The application of DoE in chromatographic optimization has been widely reported and recognized for enhancing method understanding and robustness, as discussed in recent analytical reviews and studies [7–9].

Organic solvents such as methanol and acetonitrile are commonly used in reversed-phase HPLC, with their proportion significantly influencing analyte elution behavior and separation efficiency [10]. Along with solvent composition, buffer pH and flow rate are considered critical variables that must be systematically optimized to achieve consistent chromatographic performance.

A survey of the literature indicates that numerous analytical methods have been reported for the determination of citicoline alone and in combination with other drugs. These include UV spectrophotometric methods such as difference spectroscopy [11], first-order derivative spectroscopy [12], calibration curve methods [13], as well as chromatographic and electrochemical techniques including gas chromatography [14], potentiometric transduction [15], HPLC analysis in human plasma [16], pharmacokinetic studies [17], conventional HPLC methods [18], and stability-indicating HILIC methods [19]. Simultaneous estimation of citicoline with other drugs has also been reported using UV absorption correction methods, Q-absorbance methods [20], RP-HPLC [21], UPLC [22], and HPTLC techniques [23].

Similarly, several analytical approaches have been described for the determination of nimodipine, both individually and in combination with other pharmaceutical agents. These include UV spectrophotometric methods such as calibration curve techniques [24], zero-order and derivative spectroscopy [25], solvent-based UV methods [26], colorimetric analysis [27], HPLC with UV detection [28], impurity profiling by HPLC [29], QbD-based impurity determination [30], LC-MS and LC-MS/MS methods for human plasma analysis and pharmacokinetic applications [31,32]. A few studies have reported simultaneous RP-HPLC determination of citicoline and nimodipine, including stability-indicating methods in the presence of degradation products [33–35].

Despite these reports, there is limited literature describing the application of DoE-based RP-HPLC methods for this drug combination with explicit design space establishment, and UV spectrophotometric analysis using the simultaneous equation approach remains scarcely explored. Furthermore, no combined monograph for citicoline and nimodipine is currently available in major pharmacopoeias, highlighting the need for analytically robust and practically applicable methods.

In this context, the present study aims to develop and validate two complementary analytical methods: a UV spectrophotometric method based on the simultaneous equation approach, and an RP-HPLC method optimized using a QbD-guided Central Composite Design. The objective is to achieve reliable, accurate, and efficient simultaneous determination of citicoline and nimodipine in synthetic pharmaceutical formulations, with particular emphasis on method understanding, robustness, and suitability for routine quality-control applications.

2. Experimental methods

2.1. Equipment

A Shimadzu HPLC-2030 Plus Prominence system, which was controlled by LabSolutions software (Shimadzu, Japan), was used for the chromatographic analysis. It had a degasser and an auto-sampler photodiode array (PDA) detector. The UV spectroscopic measurements were performed using a Shimadzu UV-1100 series spectrophotometer. Sample sonication was performed using a Labman Ultra Sonicator, and precise weighing of standards and samples was carried out using a Mettler-Toledo XPE205 analytical balance (Columbus, Ohio, USA).

2.2. Software

Method optimization was conducted using Design-Expert® software (Stat-Ease Inc., Minneapolis, USA) based on the principles of Design of Experiments.

2.3. Chemicals and reagents

Citicoline and nimodipine active pharmaceutical ingredients (APIs) were kindly provided by The Madras Pharmaceuticals, Chennai, India. A marketed tablet formulation, Nimodilat-Plus (containing citicoline 100 mg and nimodipine 30 mg), was procured from a local pharmacy for analytical evaluation. Merck, located in Mumbai, India, supplied analytical grade disodium hydrogen phosphate, sodium dihydrogen phosphate, orthophosphoric acid, and HPLC-grade methanol. Every solvent and reagent used was analytical or HPLC grade.

2.4. Conditions for spectrophotometry and chromatography

The Shimadzu HPLC-2030 Plus-Prominence system, which has a photodiode array (PDA) detector, was used to perform HPLC analysis. On a Hypersil C18 column (250 mm × 4.6 mm, 5 µm particle size), chromatographic separation was achieved. Methanol (40%) and

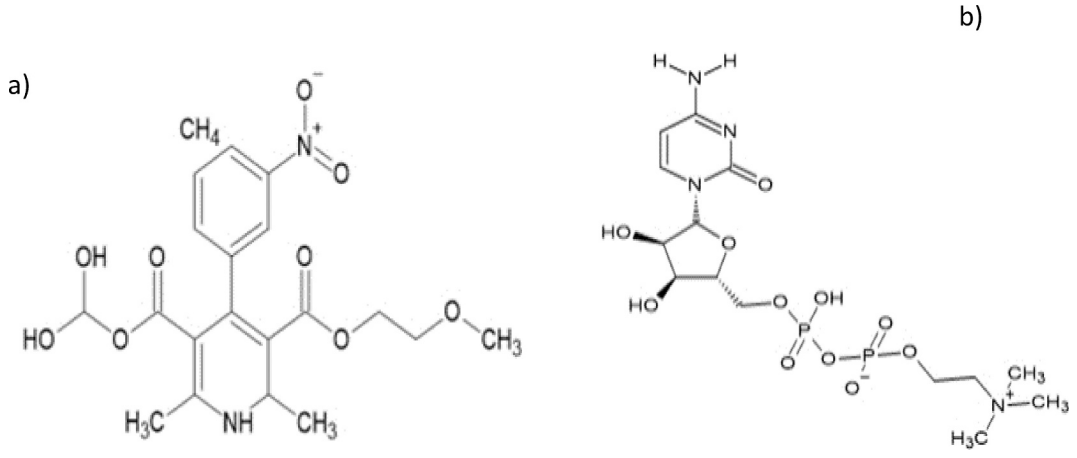


Fig. 1. Chemical structures of Citicoline (a), Nimodipine (b).

phosphate buffer (60%) that had been pH 3.8 adjusted with ortho phosphoric acid made up the mobile phase. The mobile phase was supplied at a flow rate of 1.0 mL/min after being degassed and filtered through a 0.45 μm membrane filter. A PDA detector was used for detection, and the injection volume was set at 20 μL.

For UV spectrophotometric analysis, a Shimadzu UV-1100 series spectrophotometer was used. The solvent utilized to make the samples and standards were methanol. Absorbance measurements for citicoline and nimodipine were recorded at their respective wavelengths, using methanol as the blank. Based on the overlaid UV spectra of both analytes, a common detection wavelength of 225 nm was selected for RP-HPLC analysis to ensure adequate sensitivity for both drugs.

2.5. Analytical target profile (ATP)

The Analytical Target Profile (ATP) for the RP-HPLC method was defined to achieve adequate resolution between citicoline and nimodipine within a short run time, with acceptable peak symmetry and reproducibility suitable for routine quality-control analysis.

2.6. Standard stock solution preparation

30 mg of nimodipine and 100 mg of citicoline were precisely weighed to produce the primary stock solutions and dissolving each in 100 mL volumetric flasks with methanol, resulting in concentrations of 300 μg/mL for nimodipine and 1000 μg/mL for citicoline. For UV spectroscopic method, 0.9 mL of the primary stock solution after transferring the stock solution into a 100 mL volumetric flask, it was diluted with methanol to achieve a final concentration of 9.0 μg/mL for citicoline and 2.7 μg/mL for nimodipine.

For RP-HPLC method, the standard solution for chromatographic analysis, 1.5 mL of the primary stock solution was diluted with the mobile phase up to 10 mL, resulting in 45 μg/mL citicoline and 15 μg/mL nimodipine. This solution was used for subsequent chromatographic evaluation.

2.7. Sample solutions preparation

Ten Nimodilat-Plus tablets, each containing 30 mg of nimodipine and 100 mg of citicoline, were weighed to obtain the average tablet weight. A quantity of tablet powder equivalent to one tablet (containing 30 mg nimodipine and 100 mg citicoline) was transferred into a 100 mL volumetric flask. Approximately 50 mL of methanol was added, and the mixture was sonicated for 10 min to ensure complete dissolution. The solution was then made up to volume with methanol and filtered through Whatman filter paper No. 41 (0.45 μm pore size).

For UV spectrophotometric analysis, 0.9 mL of the filtered solution was diluted to 100 mL with methanol to achieve final concentrations of 2.7 μg/mL nimodipine and 9 μg/mL citicoline. For HPLC analysis, 1.5 mL of the filtered sample solution was diluted to 10 mL with the mobile phase, yielding concentrations of 15 μg/mL nimodipine and 45 μg/mL citicoline. This solution was used for subsequent chromatographic evaluation.

2.8. System suitability studies

System suitability was evaluated prior to analysis by assessing theoretical plates, tailing factor, and capacity factor. All parameters complied with USP and ICH Q2 (R1) requirements, confirming system adequacy.

2.9. Preparation of calibration graph

For the UV spectrophotometric method, working solutions were prepared from primary stock solutions of citicoline (1000 μg/mL) and nimodipine (300 μg/mL). Final concentrations of 2.1–3.6 μg/mL for nimodipine and 7–12 μg/mL for citicoline were obtained by transferring aliquots ranging from 0.7 to 1.2 mL into 100 mL volumetric flasks and diluting them with methanol. Plotting absorbance against concentration allowed for the creation of calibration curves. Absorbance was measured at each wavelength.

For the RP-HPLC method, final concentrations of 15–90 μg/mL for citicoline and 5–30 μg/mL for nimodipine were obtained by diluting aliquots of 0.5–3.0 mL from the stock solutions with mobile phase in 100 mL volumetric flasks. Chromatograms were recorded at 225 nm following the injection of 20 μL of each solution. Plotting peak area against concentration allowed for the creation of calibration curves. All measurements were performed in triplicate, and the linearity range conformed to Beer–Lambert's law.

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

The LOQ and LOD for both methods were determined using the calibration curve approach. These were calculated using the standard deviation of the response (σ) and the slope (S) of the calibration curve, using the following equations:

$LOQ = 10 \times (\sigma/S)$

$LOD = 3.3 \times (\sigma/S)$

Linearity studies were performed in triplicate to ensure reliability and reproducibility of the estimated limits.

2.9.2. Precision

Precision was evaluated by conducting repeatability studies on both UV and HPLC methods. Six replicate determinations were carried out at the same concentration level for each method. The results were expressed as % RSD. Values within the acceptable limit (typically Relative Standard Deviation $<2\%$) confirmed the precision of the methods.

2.9.3. Accuracy

Accuracy was assessed by performing recovery studies in accordance with ICH guidelines. Tablet powder equivalent to 100 mg of citicoline was spiked with standard drug solutions at three concentration levels: 50%, 100%, and 150% of the nominal concentrations. The spiked samples were filtered using Whatman filter paper No. 41 after being sonicated for 15 min, and then further diluted as necessary for HPLC and UV analysis. Each recovery level was analyzed in triplicate, and the % RSD and percentage recovery were calculated. Low % RSD values and recovery values between 98 and 102% attested to the precision and dependability of the suggested techniques.

2.10. Ruggedness

Ruggedness was evaluated by repeating the analysis under identical experimental conditions by a different analyst on a different day. The drug content and %RSD values were compared across analysts. The minimal variation observed confirmed the inter-analyst reproducibility and ruggedness of both UV and RP-HPLC methods.

2.11. Justification for Selection of DoE Variables and Ranges

The selection of critical method parameters and their experimental ranges was based on preliminary trials and the physicochemical properties of citicoline and nimodipine. The methanol concentration range was selected to ensure adequate retention and resolution while maintaining reasonable analysis time. The pH range (3.8–4.2) was chosen to maintain analyte stability and acceptable peak symmetry, particularly for nimodipine, while minimizing excessive variability in retention behavior. The flow rate range (0.8–1.2 mL/min) represents typical operational variability and was selected to evaluate method robustness within practical chromatographic limits.

3. Results and discussion

3.1. Validation of the UV spectrophotometric method

3.1.1. Linearity

As shown in "Fig. 2", the overlay spectra of citicoline and nimodipine confirmed well-defined absorbance peaks at their respective wavelengths. For citicoline and nimodipine, the method showed good linearity over concentration ranges of 7–12 μg/mL and 2.1–3.6 μg/mL, respectively. Curves of calibration exhibited strong correlation coefficients ($R^2 > 0.999$), indicating excellent linear relationships between absorbance and concentration for both analytes.

3.1.2. LOD and LOQ

The response standard deviation and the calibration curve slope were used to calculate the limits of detection and quantification. Nimodipine and citicoline were found to have respective limits of detection of 0.0893 μg/mL and 0.4297 μg/mL. The corresponding quantification values for citicoline and nimodipine were 1.3022 μg/mL and 0.2706 μg/mL, respectively. The sensitivity and suitability of the method for detecting low analyte concentrations are confirmed by these low values.

3.1.3. Precision

Repeatability studies (intra-day precision) were performed at a single concentration level ($n = 6$). The UV method high precision was demonstrated by the percentage RSD values of 0.9974% for nimodipine and 1.0226% for citicoline, both of which fell within the acceptable range (less than 2 percentage).

3.1.4. Accuracy

Recovery studies were used to evaluate accuracy by spiking previously examined samples with known quantities of citicoline and nimodipine at 50, 100, and 150% levels. The percentage recoveries were 99.88% for citicoline and 100.35% for nimodipine, with corresponding % RSD values of 0.1390% and 0.0918%, respectively. These results indicate the absence of interference from tablet excipients and confirm the method reliability for accurate quantification.

3.1.5. Ruggedness

Two independent analysts conducted the analysis on separate days to confirm the UV method robustness. Percentage RSD values for nimodipine were 1.3727% (Analyst 1) and 1.4332% (Analyst 2), while for citicoline, they were 1.6158% and 0.7703%, respectively. These low % RSD values demonstrate that the method is rugged and reproducible under variable conditions. A comprehensive summary of UV method validation parameters is provided in Table 1.

3.2. Quality by Design (QbD) method optimization

A chemometric technique utilizing Response Surface Methodology (RSM) and Derringer's desirability function was used to improve the performance of chromatography. The integration of these tools allowed for the systematic optimization of key method variables to achieve improved resolution, optimal retention time, and selective analyte separation.

A Central Composite Design (CCD) was used to optimize the process, enabling the evaluation of the interactive effects among critical chromatographic variables. The study successfully identified and optimized three Critical Method Parameters (CMPs):

- Methanol concentration (A): 40–65% *v*/v
- Buffer pH (B): 3.8–4.2
- Flow rate (C): 0.8–1.2 mL/min

Table 1
Validation parameters report for UV method.

Parameters		Nimodipine	Citicoline
Beers Law limit (μg / mL)		2.1–3.6	7–12
Correlation Coefficient (r)		0.9990	0.9995
Regression equation (y = mx + c)		y = 0.0986× + 0.0778	y = 0.0169× + 0.0934
Slope(m)		0.0986	0.0169
Intercept(c)		0.0778	0.0934
LOD (μg / mL)		0.0893	0.4297
LOQ (μg/ mL)		0.2706	1.3022
Precision (% RSD)		0.9974	1.0226
Assay (%)		100.35	99.88
Accuracy (% RSD)		0.0918	0.1390
Ruggedness (% RSD)	Analyst - 1	1.3727	1.6158
	Analyst - 2	1.4332	0.7703

% RSD- Percentage Relative Standard Deviation, LOD- Limit of Detection, LOQ-Limit of Quantification.

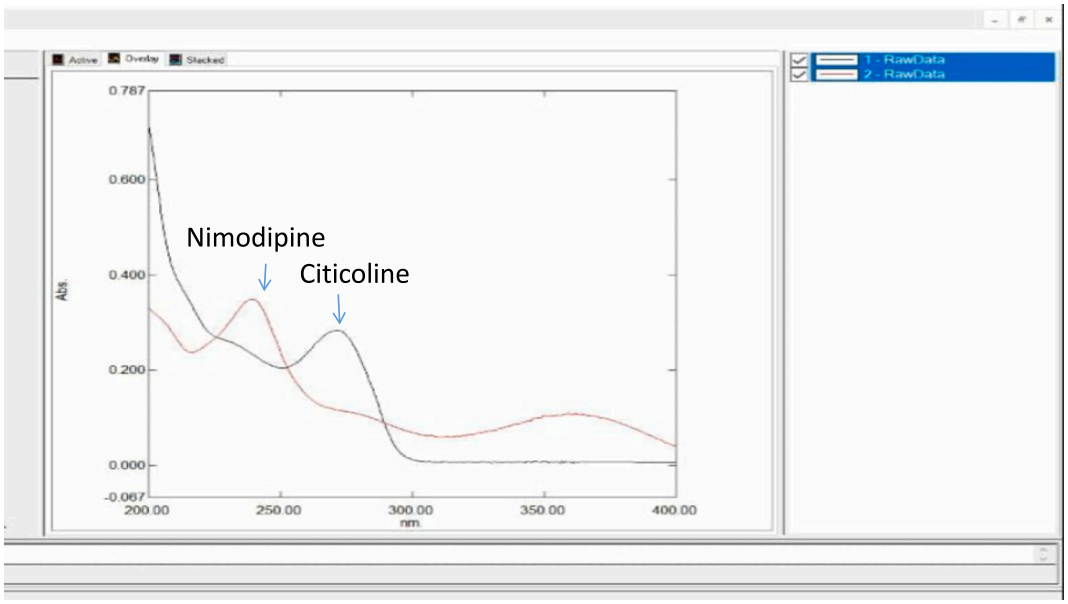


Fig. 2. Overlay UV spectrum for citicoline and nimodipine.

In accordance with ICH Q8(R2) and Q9 guidelines, the optimization process was directed by QbD principles. A risk assessment was performed to prioritize parameters based on their potential impact on method performance. This structured approach ensured a robust, reliable, and well-controlled chromatographic method.

The critical responses monitored during the optimization included

- Retention time of citicoline (Rt_1)
- Capacity factor of citicoline (K_1)
- Resolution between citicoline and nimodipine peaks ($Rs_{1,2}$)

The use of CCD allowed for systematic modulation of the chromatographic conditions and provided a deeper understanding of how the CMPs interact to influence separation efficiency.

A control strategy, a crucial element of QbD, was integrated into the approach after method development. The first step involved defining an Analytical Target Profile (ATP), which served as the foundation for aligning method development with desired performance outcomes. This strategy included predefined controls based on risk management and ensured fitness for intended purpose.

The approach also followed ICH Q8(R2)'s definition of CQAs, which are in accordance with QbD practices. CQAs are attributes chemical, biological, microbiological, or physical that need to be kept within predetermined bounds in order to guarantee the quality of the final product. CQA identification and control were guided by the Quality Target Product Profile connecting analytical method development to broader product and process understanding.

Method ruggedness was assessed through inter-analyst and inter-day variability studies, as described in the validation section.

A summary of the Central Composite Design (CCD) layout and experimental responses is provided in Table 2.

3.3. Experimental design and statistical modeling

To minimize the influence of uncontrolled variables and reduce experimental bias, all experimental trials were executed in a randomized sequence. Additionally, six center point replicates ($n = 6$) were included in the design to evaluate experimental error and assess the reproducibility of the method.

A three-factor CCD was applied to explore the impacts of critical chromatographic specifications:

- **X_1**: Methanol concentration (A)
- **X_2**: Buffer pH (B)
- **X_3**: Flow rate (C)

The connection across the input factors and output responses was explained by means of a second-order polynomial equation that contained interaction, linear, and quadratic terms:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X2 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32}.$$

Where:

- **Y** is the expected reaction (e.g., retention time, resolution, or capacity factor),
- **β_0** is the intercept,
- **β_1 to β_3** are the linear coefficients,
- **β_{12}, β_{13}, β_{23}** are the interaction coefficients,
- **β_{11}, β_{22}, β_{33}** are the quadratic coefficients,
- **X_1, X_2, X_3** The coded values of the independent variables are represented.

This model enabled the prediction and interpretation of the influence of experimental variables on key chromatographic responses. The model adequacy and statistical significance were assessed by Analysis of Variance (ANOVA). The reduced models, obtained by eliminating statistically insignificant terms, are detailed with their respective statistical parameters in Table 3.

3.4. Model reduction, interpretation, and optimization

To enhance the model's predictive power and practical applicability, backward elimination was used to eliminate terms that were statistically

Table 3
Reduced Response Surface Models and Statistical Parameters Obtained from ANOVA.

Responses	Adjusted R2	Model *p* value	% C.V	Adequate Precision
K1	0.8655	<0.0001	3.97	6.8382
Rt1	0.8223	<0.0001	4.94	4.2270
Rs1,2	0.8431	<0.0001	4.73	9.0341

%CV- Percentage Co-efficient of Variation.

Table 2
Central composite arrangement and responses.

Run	Space type	Methanol Conc. (%v/v)	Buffer pH	Flow rate (mL /min)	Capacity factor (K_1)	Retention time (Rt_1)	Resolution ($Rs_{1,2}$)
7	Center	60	4	1	1.2	3	3.7
10	Center	60	4	1	1.2	3	3.7
12	Center	60	4	1	1.2	3	3.7
17	Center	60	4	1	1.2	3	3.7
18	Center	60	4	1	1.2	3	3.7
19	Center	60	4	1	1.2	3	3.7
2	Axial	60	3.66	1	1.14	3.52	2.860
4	Axial	60	4	1.33	1.3	4.02	4.239
13	Axial	68.409	4	1	0.8	4.01	8.208
15	Axial	51.591	4	1	1.14	4.49	1.896
16	Axial	60	4	0.66	1.2	4.05	4.85
20	Axial	60	4.33	1	1.25	6.05	5.583
1	Factorial	65	3.8	1.2	1.13	5.27	8.836
3	Factorial	55	3.8	1.2	1.12	3.54	9.423
5	Factorial	65	4.2	0.8	0.98	3.28	7.012
6	Factorial	55	4.2	1.2	1.23	5.38	6.019
8	Factorial	65	4.2	1.2	1.16	4.11	5.112
3	Factorial	55	4.2	0.8	1.24	4.91	3.98
2	Factorial	65	3.8	0.8	1.15	5.78	3.123
1	Factorial	55	3.8	0.8	1.17	4.72	3.45

Conc% V/V - Concentration Percentage Volume/ Volume, Min - Minutes, Rt1-Retention of first peak (citicoline), K1 - Capacity factor of the first peak (Citicoline), Rs1,2- Resolution between the two peaks (Citicoline and Nimodipine).

non-significant ($p > 0.05$). The final models retained only those terms with p-values <0.05, indicating statistical significance.

The adjusted R^2 values of the reduced models indicated a good fit between the experimental data and the second-order polynomial equations, confirming that the models effectively described the system's behavior while accounting for the number of regressor terms.

All reduced models exhibited adequate precision (signal-to-noise ratio) values well above the minimum threshold of 4, ranging from 4.2270 to 9.0341, confirming that the models provided sufficient signal to allow reliable navigation of the design space [36].

The coefficient of variation (C·V.) values were all below 10%, indicating excellent model reproducibility and reliability across experimental replicates [37].

The regression coefficients obtained from the polynomial models provide insight into the chromatographic behavior of citicoline and nimodipine. An increase in methanol concentration resulted in a decrease in the capacity factor due to enhanced elution strength of the mobile phase, which reduces analyte–stationary phase interactions. However, resolution improved at intermediate methanol levels, indicating an optimal balance between retention and selectivity. Buffer pH significantly influenced peak symmetry and resolution, reflecting changes in analyte ionization and silanol interactions on the stationary phase. Flow rate exhibited a negative effect on resolution at higher levels due to reduced analyte interaction time with the stationary phase. These observations are consistent with fundamental chromatographic principles and confirm the suitability of the selected design space.

3.5. Mathematical Models and Interpretation

3.5.1. Capacity Factor (K_1)

$$K_1 = +1.20 - 0.0668^{*}A + 0.0165^{*}B + 0.0196^{*}C - 0.0400^{*}AB + 0.0275^{*}AC + 0.0300^{*}BC - 0.0786^{*}A^2 + 0.0010^{*}B^2 + 0.0204^{*}C^2 \quad (1)$$

Eq. (1) suggests that:

Increasing methanol concentration (A) decreases the capacity factor. Increasing flow rate (C) and buffer pH (B) rise the capacity factor.

This behavior aligns with the general chromatographic principle that higher organic content reduces analyte interaction with the stationary phase, thereby lowering retention and capacity.

3.5.2. Retention Time (Rt_1)

$$Rt_1 = +2.99 - 0.0672^{*}A + 0.1922^{*}B - 0.0323^{*}C - 0.7113^{*}AB + 0.1287^{*}AC + 0.3738^{*}BC + 0.4809^{*}A^2 + 0.6701^{*}B^2 + 0.4049^{*}C \quad (2)$$

From Eq. (2), it is evident that:

Methanol concentration (A) and flow rate (C) both have negative effects on retention time. Buffer pH (B) has a positive effect, increasing the retention time.

Lower methanol levels (weaker elution strength) and higher pH enhance the interaction of ionizable compounds with the stationary phase, thus prolonging retention.

3.5.3. Resolution ($Rs_{1,2}$)

$$Rs_{1,2} = 5.66 + 0.8204^{*}A + 0.0361^{*}B + 0.5986^{*}C + 0.4578^{*}AB - 0.6028^{*}AC - 1.12^{*}BC + 0.7693^{*}A^2 + 0.4757^{*}B^2 + 0.5899^{*}C^2 \quad (3)$$

Eq. (3) highlights that:

Methanol concentration (A) and flow rate (C) significantly improve resolution. The largest interaction term is AB (+0.4578), indicating a synergistic effect between methanol concentration and buffer pH on resolution. At extreme methanol concentrations, increasing pH tends to reduce resolution, suggesting the need to carefully balance both factors [34].

Graphical Interpretation. To visually understand variable influence, perturbation plots "Fig. 3a–3c" and 3D response surface plots "Fig. 4a–4c" were constructed. Perturbation plots illustrate how each individual variable affects the response when others are held constant at the reference level, while response surface plots offer a 3D visualization of variable interactions and their collective impact on the response.

Global Optimization Using Derringer's Desirability Function. To identify optimal chromatographic conditions, Derringer's desirability

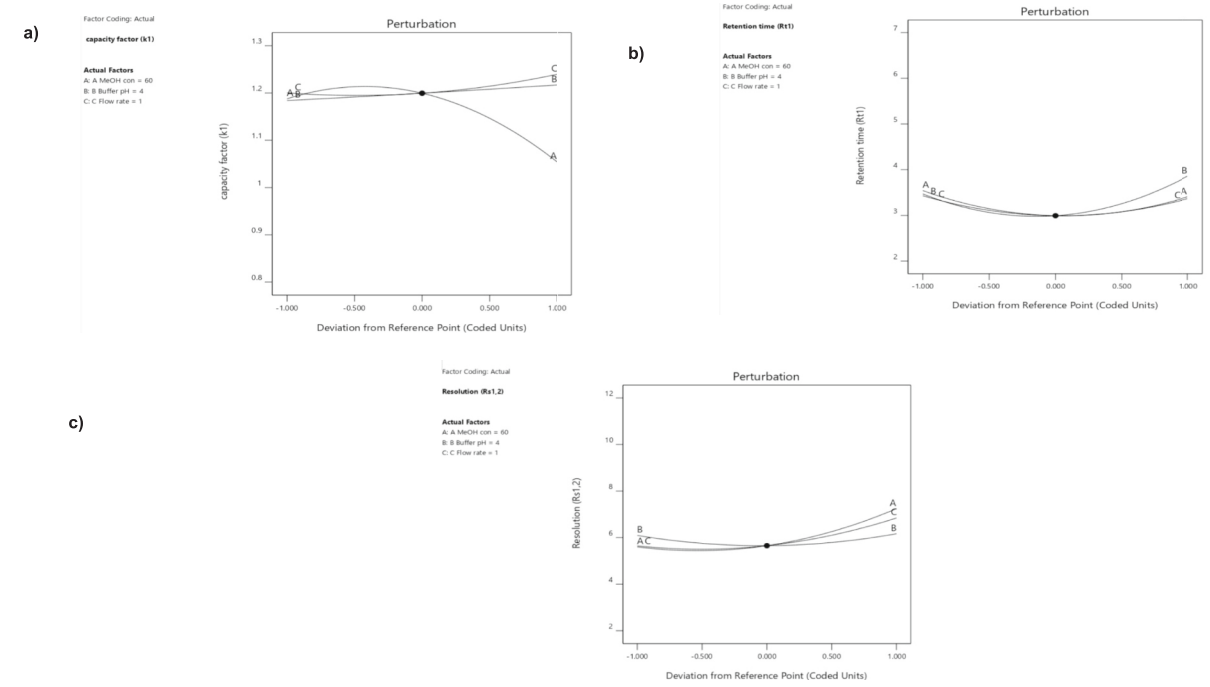


Fig. 3. (a) Perturbation plot showing the effect of methanol concentration, buffer pH, and flow rate on Capacity Factor (k_1) for first peak (Citicoline). (b). Perturbation plot depicting the effect of chromatographic variables on Retention Time (Rt_1) of first peak (citicoline). (c). Perturbation plot depicting the effect of chromatographic parameters on Resolution (Rs1,2) between citicoline and nimodipine.

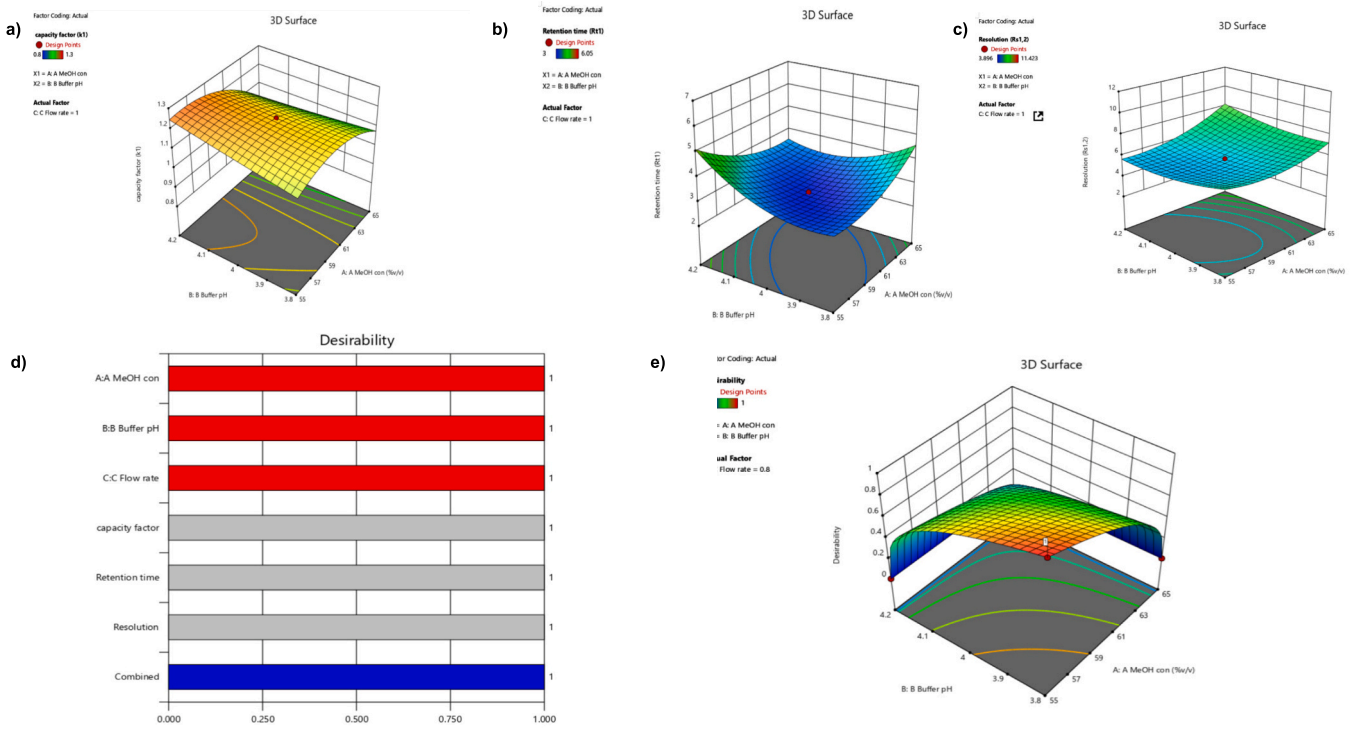


Fig. 4. (a) 3D Surface Plot Depicting the Effect of Methanol Concentration and Buffer pH on Capacity Factor (k_1) for first peak (Citicoline) at Constant Flow Rate (b) 3D Surface Plot Illustrating the Effect of Methanol Concentration and Buffer pH on Retention Time (Rt_1) for first peak (Citicoline) at constant Flow Rate (c) 3D Surface Plot Showing the Effect of Methanol Concentration and Buffer pH on Resolution ($Rs_{1,2}$) between citicoline and nimodipine peaks at constant Flow 4(d). Individual and combined Desirability scores of chromatographic parameters. (e) 3D Surface plot showing effect of methanol concentration and buffer pH on desirability at constant Flow Rate.

function was used to optimize a multi-response. Which integrates individual desirability functions for multiple responses:

$D = [d_1^{p2} \times d_2^{p2} \times d_3^{p2} \times \ldots\ldots\ldots \times d_n^{pn}]^{1/n}$.

Where:

- **D** is the overall desirability (ranging from 0 = least desired to 1 = most desired),
- $\mathbf{d_i}$ is the individual desirability for each response (e.g., retention time, resolution, capacity factor),
- $\mathbf{p_i}$ is the assigned weight reflecting the importance of each response.

In this study, resolution, capacity factor, and retention time were simultaneously optimized. Weights were selected to prioritize resolution, while minimizing run time and maintaining an acceptable capacity factor. The derived desirability function guided the selection of the optimal chromatographic conditions within the design space.

Optimization Outcome and Desirability Function. The optimization criteria were designed to balance method performance across multiple responses. Specifically:

- The citicoline retention time (tR_1) and there were restrictions on the resolution between the nimodipine and citicoline peaks ($Rs_{1,2}$) within desirable limits to ensure rapid analysis without compromising peak separation.
- The capacity factor of citicoline (K_1) was minimized to ensure adequate separation from the solvent front, improving the clarity of early-eluting peaks.

The optimization was carried out under these defined constraints using Derringer's desirability function, which integrates individual desirability values into a single, composite score reflecting overall method performance. The global desirability response surface obtained is shown in 'Fig. 4d, e", illustrating the optimal region within the design space where all critical responses meet their target values.

The criteria used to optimize each individual response are detailed in Table 4, including goal direction (maximize/minimize/target), acceptable limits, and relative importance.

Method Validation and Confirmation of Ideal Conditions. As illustrated in "Fig. 4d", Response surface methodology and desirability analysis were employed to evaluate the combined influence of methanol concentration, buffer pH, and flow rate on critical chromatographic responses. Although numerical optimization indicated regions of high desirability within the experimental domain, a mobile phase composition of methanol: phosphate buffer (40,60, *v*/v) at pH 3.8 and a flow rate of 1.0 mL/min was selected as the final operating condition based on a balanced compromise between resolution, retention behavior, peak symmetry, and method robustness. Under these conditions, a high overall desirability value (D = 0.973) was obtained, confirming that the selected conditions lie well within the established design space and provide reliable chromatographic performance. Under these optimized conditions, the predicted chromatographic responses were:

- **tR_1** (citicoline retention time) = **3.23 min**
- **k_1** (capacity factor) = **1.19**
- **$Rs_{1,2}$** (resolution between citicoline and nimodipine) = **4.775**

Table 4
Criteria for the optimization of the individual responses.

Response	Lower limit	Upper limit	Criteria/Goal
k_1	0.8	1.2	Minimize
$Rs_{1,2}$	3.86	11.42	Minimize
Rt_1	3.0	6.05	Minimize

K1- Capacity factor of the first peak (Citicoline), Rs1,2- Resolution between the two peaks (Citicoline and Nimodipine).

To verify model reliability, an experiment was performed under these ideal settings. The obtained chromatogram is shown in "Fig. 5". The predictive accuracy of the model was evaluated by calculating the percentage prediction error for each response using the formula:

Prediction error = Experimental- Predicted/ Predicted.

The prediction error values are summarized in Table 5, confirming close agreement between experimental and predicted results, and confirming the developed method adaptability.

Method Validation HPLC. The developed RP-HPLC method demonstrated simplicity, accuracy, and precision in the simultaneous estimation of nimodipine and citicoline. The validation parameters, summarized in Table 6, conform to ICH Q2 (R1) guidelines.

The method exhibited excellent linearity in the range from 15 to 90 µg/mL for citicoline and 5–30 µg/mL for nimodipine with correlation coefficients (R^2) consistently above 0.999.

The sensitivity of the method was demonstrated by the determination of the quantification limit at 2.0856 µg/mL and 1.1233 µg/mL, respectively, and the detection limit at 0.6882 µg/mL for nimodipine and 0.3707 µg/mL for citicoline. Precision studies showed % RSD values of 0.7324 for nimodipine and 1.4741 for citicoline, both well below the acceptable threshold of 2%, confirming the method repeatability and reliability. The method also displayed high accuracy, with recovery values for an average percentage of 100.61% for nimodipine and 100.41% for citicoline. The % RSD values for recovery were 0.7082 and 0.9599, respectively, further supporting the robustness and reproducibility of the method.

These outcomes demonstrate that the developed RP-HPLC method is appropriate for regular quality control analysis of citicoline and nimodipine in pharmaceutical dosage forms.

Statistical Comparison of UV and RP-HPLC Methods. To assess the equivalence of the developed UV spectroscopic and RP-HPLC methods, six samples from two independent batches were analyzed using both techniques. To identify any notable variations in the estimation of citicoline and nimodipine, the data were statistically assessed at a 95% confidence level ($P = 0.05$). Both the method effect and the method–sample interaction were examined separately using a two-way ANOVA [35]. The computed F-statistic (F_{stat}) in both instances was less than the critical F-value (Fcrit), suggesting that there were neither sample-method interactions nor significant differences between the two methods Table 7 and Table 8.

Furthermore, the mean values obtained from UV and HPLC analyses was compared using a paired Student's *t*-test. Both techniques produce comparable and trustworthy results for the simultaneous quantification of citicoline and nimodipine, according to the results, which showed no statistically significant differences between the techniques. These results support the use of either technique for regular quality control and validate the inter-method consistency.

Table 5
Comparison of experimental and predictive values of different functions under optimal conditions.

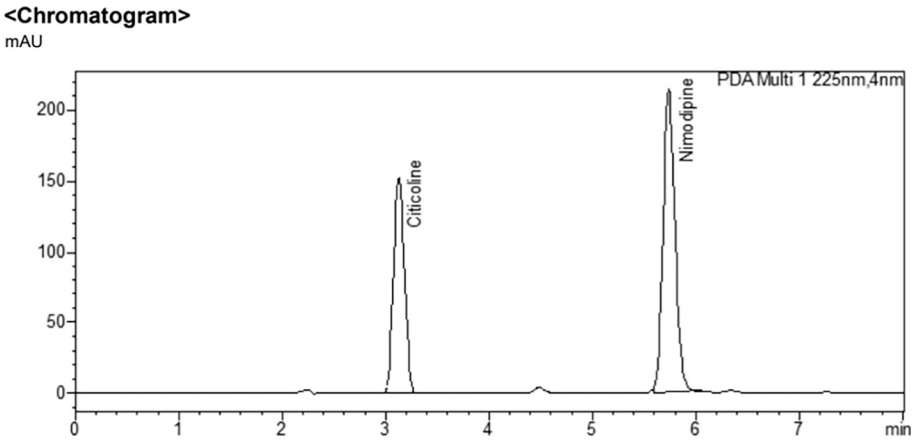
Optimum conditions	MeOH Conc. (% v/v)	Buffer pH	Flow rate (mL /min)	Capacity Factor (k1)	$Rs_{1,2}$	Rt_1
Predictive	55.00	3.8	0.8	1.50	4.775	3.15
Experimental	55.00	3.8	0.8	1.59	4.841	3.23
Average error				4.021	1.787	2.539
Desirability value (D) =0.974						

K1- Capacity factor of the first peak (Citicoline), Rs1,2- Resolution between the two peaks.
(Citicoline and Nimodipine)

Table 6
Validation parameters Report for RP-HPLC method.

Parameters		Nimodipine	Citicoline
Beers Law limit (µg/ml)		5–30	15–90
Correlation Coefficient (r)		0.9997	0.9995
Regression equation (Y = mx + c)		y = 177.75× +13,543	y = 1019.5× + 78,118
Slope (m)		177.75	1019.5
Intercept(c)		13,543	78,118
LOD (µg/ml)		0.6882	0.3707
LOQ (µg/ml)		2.0856	1.1233
Precision (%RSD)		0.7324	1.4741
Assay (%)		*100.35*	99.88
Accuracy (%RSD)		0.7082	0.9599
Ruggedness	Analyst -I	1.0259	0.9314
	Analyst-2	0.9629	0.2125

% RSD- Percentage Relative Standard Deviation, LOD- Limit of Detection, LOQ- Limit of Quantification.



<Peak Table>
PDA Ch1 225nm

Peak#	Ret. Time	Area	Conc.	Unit	Mark	Name
1	3.006	124655	0.000			
2	5.739	16229	0.000			
Total		140884				

Fig. 5. Optimized RP-HPLC chromatogram of Citicoline and Nimodipine at 225 nm with Retention Times of 3.006 min and 5.739 min, respectively.

Table 7
Two-way ANOVA Test of citicoline and nimodipine in six-independent samples by UV and HPLC.

Two way ANOVA Test of citicoline determination				
HPLC[a]			UV[a]	
Sample	First sampling	Second sampling	First sampling	Second sampling
1	100.11	100.52	100.23	100.26
2	100.56	100.34	99.45	99.58
3	99.23	99.99	99.98	100.45
4	100.24	100.17	100.32	99.32
5	100.36	99.64	99.52	100.06
6	99.98	99.32	99.9	99.56

ANOVA: Two-Factor with Replication						
Source of Variation	SS	df	MS	F	*p*-value	F crit
Sample	0.139537	1	0.139537	0.750077	0.396723	4.351244
Columns	0.018704	1	0.018704	0.100543	0.754465	4.351244
Interaction	0.004537	1	0.004537	0.024391	0.87746	4.351244
Within	3.720617	20	0.186031			
Total	3.883396	23				

Two way ANOVA Test of nimodipine Determination				
HPLC[b]			UV[b]	
Sample	First sampling	Second sampling	First sampling	Second sampling
1	30.11	30.53	30.49	30.89
2	30.22	30.64	30.65	30.67
3	30.25	30.65	30.69	30.71
4	30.62	30.71	30.78	30.98
5	30.59	30.78	30.63	30.69
6	30.82	30.89	30.98	30.89

ANOVA: Two-Factor with Replication.						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0.209067	1	0.209067	6.042098	0.023216	4.351244
Columns	0.201667	1	0.201667	5.828236	0.025476	4.351244
Interaction	0.040017	1	0.040017	1.156495	0.294996	4.351244
Within	0.692033	20	0.034602			
Total	1.142783	23				

[b]The results are presented as mg of label claim amount of nimodipine in tablet.
SS-Sum of Square, df- degrees of freedom, MS- Mean Square.
SS-Sum of Square, df- degrees of freedom, MS- Mean Square.
[a] The results are presented as mg of label claim amount of Citicoline in tablet.

4. Comparison with reported methods

As summarized in Table 9, the developed RP-HPLC method demonstrated greater sensitivity than previously reported methods, as evidenced by its lower detection limit and quantification limit for both citicoline and nimodipine. These findings indicate that the developed RP-HPLC method offers improved sensitivity and analytical efficiency while remaining suitable for routine quality-control applications. Although previously reported methods offered a broader linearity range, the developed method exhibited shorter retention times for both analytes, thereby enhancing analytical efficiency and reducing overall run time. Because of this attribute, the technique is ideal for high-throughput analysis in standard quality control environments. Moreover, due to its high sensitivity, the developed method requires only minimal sample quantities, offering an economical advantage in analytical laboratories. These results demonstrate improved sensitivity and analytical efficiency while maintaining suitability for routine quality-control applications.

5. Conclusion

Two analytical methods were developed and validated in this study as the primary goal: (i) ultraviolet spectroscopy using the simultaneous equation method, and (ii) RP-HPLC based on a Quality by Design (QbD) approach for the concurrent estimation of citicoline and nimodipine in pharmaceutical formulations.

Both techniques were rigorously validated in accordance with ICH Q2 (R1) guidelines and demonstrated excellent accuracy, precision, and reliability. The UV method was found to be simple, rapid, and economical, making it highly suitable for routine applications.

For the HPLC method, a Central Composite Design (CCD) was employed to model and optimize critical variables such as buffer pH, flow rate, and methanol concentration. Derringer's desirability function was applied to identify the optimal chromatographic conditions, leading to improved separation, reduced analysis time, and a deeper understanding of variable interactions.

Statistical comparison using Student's *t*-test and two-way ANOVA revealed no significant differences between the two methods, confirming their interchangeability and robustness for routine analysis.

In conclusion, the developed UV and RP-HPLC methods are efficient,

Table 8
Determination of HPLC and UV and their correlation by paired t-Test.

Student t-Test for Citicoline		
Sample	HPLC[a]	UV[a]
1	100.51	100.65
2	100.77	100.64
3	100.73	99.79
4	100.66	99.98
5	100.56	99.99
6	100.69	100.68
Average	100.68	100.21

Student t-Test: Paired two sample for Means (Citicoline)		
Parameters	Variable 1	Variable 2
Mean	100.682	100.216
Variance	0.00637	0.17083
Observations	5	5
Pearson Correlation	0.404242	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.685712	
P(T ≤t) one-tail	0.027451	
t Critical one-tail	2.131847	
P(T ≤t) two-tail	0.054902	
t Critical two-tail	2.776445	

Student t-Test for nimodipine		
Sample	HPLC[b]	UV[b]
1	30.23	30.69
2	29.94	30.89
3	29.96	29.89
4	30.65	29.99
5	29.89	30.89
6	30.23	30.87
Average	30.13	30.50

Student *t*-Test: Paired two sample for Means (nimodipine).		
	Variable 1	Variable 2
Mean	100.134	100.506
Variance	0.10073	0.26828
Observations	5	5
Pearson Correlation	−0.44455	
Hypothesized Mean Difference	0	
df	4	
t Stat	−1.15892	
P(T ≤t) one-tail	0.155481	
t Critical one-tail	2.131847	
P(T ≤t) two-tail	0.310963	
t Critical two-tail	2.776445	

df- degrees of freedom.
[a] The results are presented as mg of label claim amount of Citicoline in tablet.
[b] The results are presented as mg of label claim amount of nimodipine in tablet.

Table 9
Comparison data between Developed and Reported method.

Parameters	Drugs	Developed method	Reported method [33]
Retention Time	Citicoline	2.548	8.103
	Nimodipine	3.241	3.888
LOD(μg / mL)	Citiocoline	0.011	1.04
	Nimodipine	0.018	16.0
LOQ (μg / mL)	Citicoline	0.034	3.4
	Nimodipine	0.055	48
Linearity (μg/ mL)	Citicoline	2–12	10–80
	Nimodipine	40–240	160–960

LOD- Limit of Detection, LOQ-Limit of Quantification.

reproducible, and reliable, making them highly appropriate for the quality control of citicoline and nimodipine in both bulk and finished pharmaceutical dosage forms.

CRediT authorship contribution statement

T. Sudha: Project administration, Methodology, Investigation, Data curation, Conceptualization. **K. Archana:** Validation, Software. **V.S. Akash Kumar:** Writing – original draft, Resources. **R. Bharath:** Writing – review & editing, Writing – original draft, Software, Resources. **J. Amsavalli:** Resources, Formal analysis.

Funding

This research received no external funding.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors are thankful to the management of Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai for providing research facilities and encouragement.

Data availability

No data was used for the research described in the article.

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