RESEARCH ARTICLE

# An Ecologically Sound RP-HPLC Method for Estimating Citicoline using AQbD with Degradation Studies

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## ABSTRACT

**Background:** Citicoline is widely used for neurological complications. This research work was carried out using reverse-phase high-performance liquid chromatography (RP-HPLC) employing green analytical chemistry principles.

**Materials and Methods:** The method was completely developed with ethanol:water (20:80 v/v) as the mobile phase, 0.1% orthophosphoric acid was used to adjust the pH to 3.5, and an Inertsil ODS 3 (250 x 4.6 mm, 5 µm) column as the stationary phase, the flow rate was set at 1-mL/min. The column temperature was maintained at 35ºC, and an injection volume of 10 µL was used for detection at a wavelength of 250 nm.

**Results and Discussion:** The peak was eluted with isocratic elution with a retention time of 3.996 minutes. The linear concentration range for this method was 10 to 50 µg/mL, and the correlation coefficient R2 was 0.999. The forced degradation studies have shown that 3.5% w/w degraded in acid, 1.35% w/w in alkali, and 0.2% w/w in oxidation. The products formed during degradation were characterized using gas chromatography-mass spectrometry (GC-MS).

**Conclusion:** The International Council for Harmonisation (ICH) guidelines were followed in the development and validation of the method optimized by the use of central composite design, and the method's greenness was assessed through the use of AGREE, AES, GAPI, and AMGS, among other green assessment tools.

**Keywords:** AES AGREE, AMGS, Central composite design, GAPI GC-MS, RP-HPLC.

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**Conflict of interest:** None

## INTRODUCTION

Parkinson's disease is a neurological disorder characterized by unintentional or uncontrollable movements such as trembling, stiffness, and difficulties with balance and coordination.[1] Usually, the onset and progression of symptoms are incremental.[2] People may experience difficulty walking and speaking as the disease progresses.[3] Citicoline is widely useful for Parkinson's disease.[4-5] Figure 1 Shows the structure of citicoline.

Citicoline is 5'-O-[hydroxy ({hydroxy [2-(trimethylammonium) ethoxy] phosphoryl} oxy) phosphoryl] cytidine.[6] It is a white, crystalline powder that dissolves readily in water but not in alcohol, acetone, or chloroform. It is often called "brain food" because it raises the number of neurotransmitters in the brain, helps maintain the integrity of neuronal cell membranes, and gives the prefrontal cortex more energy. This medicine is used to treat brain damage, cerebrovascular diseases like stroke, age-related cognitive decline, Parkinson's disease, glaucoma, and different types of dementia.[7] Citicoline is available on the market in both oral and injectable forms.[8] The drug's bioavailability is superior to that of rival medications because it functions the same whether taken orally or intravenously. The suggested method makes the development and validation of citicoline quick, accurate, and easy. It is also the most reliable method for this drug because it uses analytical quality by design with a central composite design and is employed by green analytical chemistry.

Quality by design (QbD), is the method of developing results that consistently meet defined criteria.[9] Due to its focus on risk assessment and management, the quality-by-design methodology has the potential to provide a more robust or rugged methodology.[10] QbD relies heavily on experimental evidence to determine which variables, factors, and interactions impact the desired outcomes.[11] This paper presents the method development and validation of an high-performance liquid chromatography (HPLC) technique for analyzing citicoline.

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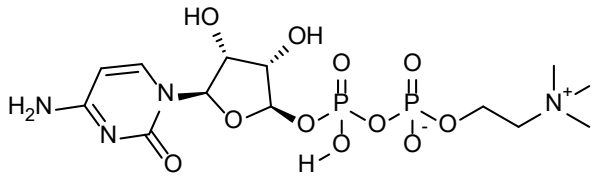


**Figure 1:** Structure of citicoline

Two designs are mainly used in QbD for the analytical method development and validation: Box Behnken design and central composite design.[12]

Using Design Expert 22.0 version[13] the central composite experimental design describes the interrelationships of mobile phase and flow rate at various levels, with retention time, area, and tailing factor as the responses to be observed. Here, a greater understanding of the factors that influence chromatographic separation is achieved, as is a higher degree of confidence in the ability of the developed HPLC method to fulfil its intended purposes. The QbD approach to analytical method development was utilized to better understand the different levels of method variables. The fundamental advantage of CCD over BBD is that it addresses the problems that arise, specifically avoiding excessive trials. An Ishikawa is used to show the different steps in a process, where quality issues may occur, and find out which supplies are needed at different times. Figure 2. shows the Ishikawa fish bone diagram.

The development of analytical techniques is one of the most active areas of R&D in green chemistry. Green analytical chemistry is a new methodology and technique that can limit and eliminate the usage and generation of hazardous compounds at all phases of chemical analysis. Toxic organic solvents, such as acetonitrile and methanol, are used. In addition to endangering the health of the researcher exposed to these solvents daily, effective waste management is required to dispose of this pollutant. Utilizing toxic solvents poses significant risks to the environment and individuals within society. It is imperative to minimize the use of these harmful solvents to mitigate their negative impacts. This method is developed and validated following the 12 green chemistry principles). The method is developed with 20% ethanol and 80% water without any buffer. Water is the most preferred green solvent and ethanol comes in second considered green solvent, even though it is employed in very small quantities. This method is completely safe, accurate, precise, robust, and ecological. The method development and validation of citicoline were previously reported without combining AQbd and green chemistry analysis.[14,15]

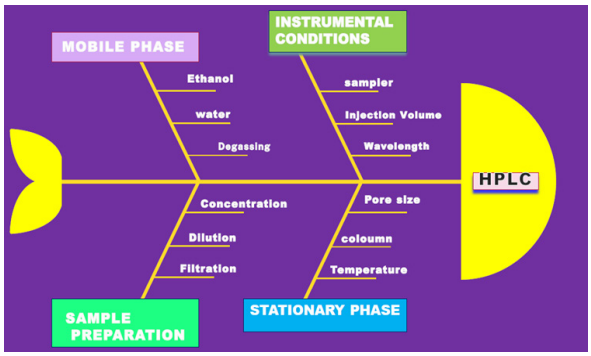



**Figure 2:** The Ishikawa fish bone diagram

## MATERIALS AND METHODS

### Chemicals and Reagents

The pure drug Citicoline was brought from (BLD Pharma Tech India Pvt. Ltd, Hyderabad, India), ethanol HPLC grade was brought from (Merck India Life Sciences), sodium hydroxide (NaOH) was brought from (Ranbaxy in New Delhi, India), conc. Hydrochloric acid (HCl) was brought from (Rankem India), orthophosphoric acid (OPA) was brought from (Yarrow chem products, Mumbai, India), 3% v/v hydrogen peroxide ($H_2O_2$) was brought from (Yarrow chem products, Mumbai, India), for HPLC grade water Milli Q filter device (ELGA Lab Water, UK) was used, 0.45 µm membrane filter was brought from (Pall life sciences Bengaluru, India) and citicoline 500 mg was brought from the nearest drugstore.

### Instrument and Software used

The Agilent 1220 Infinite II high-performance liquid chromatography (HPLC) System was utilized. The HPLC system comprises essential components like the binary pump, autosampler, and PDA detector. The software employed for HPLC analysis was Agilent Open Lab CDS chem station, specifically version 2.6. The evaluation of central composite design in Qbd Design-Expert® Software, namely version 22.0, was employed. The green analysis was conducted using the GAPI Chart Maker Version 0.1 Beta software. The AMGS tool was utilized using the AMGS calculator and the Agree Metrics were computed using the Agree tool.

### Solvents Preparation

*Preparation of standard*

The pure drug citicoline (1000 µg/mL) stock solutions were prepared in a standard flask, dissolved using water, and made up to the mark. Sonicating for 15 minutes and filtering the solution, followed by making further dilution to produce the final concentration of 10 µg/mL.

*Preparation of sample*

Take 20 citicoline tablets and use a mortar and pestle to grind them into a fine powder. Citicoline equivalent to 100 mg was precisely weighed out of a fine powder mass, transported to a 100 mL standard flask, and diluted with water to a concentration of 1-mg/mL. Then, sonicated for approximately 15 minutes, the solution and the filtrate was appropriately diluted to produce the final concentration of 10 µg/mL.

### Chromatographic Conditions

This study developed and validated the method with an HPLC Agilent 1220 Infinity II, a binary solvent delivery pump, an automatic sampling device injector, and a PDA detector. Separation was achieved by employing an Agilent Inertsil ODS

3 (250 x 4.6 mm, 5 µm). The solutions were filtered using a 0.45 µm membrane filter obtained from Pall Life Sciences in Bengaluru, India. Before use, the solvents were degassed using an ultrasonicator from Labman Maharashtra, India. The mobile phase consists of a mixture of ethanol and water, with 0.1% orthophosphoric acid used to adjust the pH of the solutions. The mobile phase consisted of ethanol and water at 20:80 v/v, and the flow rate was set at 1-mL/min. The column temperature was maintained at 35ºC, and an injection volume of 10 µL was used. The detection of the analytes was performed at a wavelength of 250 nm. The data underwent processing using the Open LAB CDS Agilent software version 2.6

**System Suitability Studies**

For the system's suitability parameters, standard citicoline solutions were injected six times. All percentage relative standard deviations (RSD) were calculated for retention time ($R_t$), peak area, and theoretical plates. Figure 3 shows the chromatogram of citicoline. Table 1 shows system suitability studies of citicoline.

**Degradation Conditions**

Citicoline stock solutions were subjected to various stress conditions explained below. Degradation was defined as a decrease in the area of the peak or the formation of new peaks. The recovery percentages determined the degree of degradation.

*Acid degradation*

For acid degradation, 0.1 M hydrochloric acid (HCl) is used. About 100 µg/mL of stock solution was transferred into 10 mL of a standard flask, then 1-mL of 0.1M HCl was added and made up with water up to the mark. It was then kept in an oven to maintain a temperature of 60ºC for 2 hours. Figure 4 Citicoline shows acid degradation.

*Alkali degradation*

For alkali degradation, 0.1 M (NaOH) is used. 100 µg/mL of stock solution was transferred into 10 mL of a standard flask,

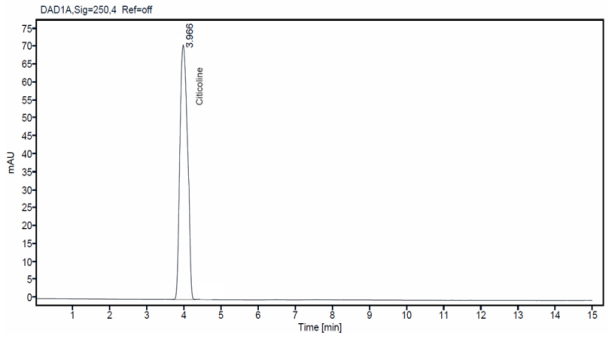



**Figure 3:** The chromatogram of citicoline

**Table 1:** System suitability studies of citicoline

| *S. No* | *Retention time* | *Tailing* | *Theoretical plates* |
|---|---|---|---|
| Mean | 3.9954 | 1.204 | 2364.9 |
| SD | 0.002 | 0.011 | 37.35 |
| %RSD | 0.07 | 0.94 | 1.57 |

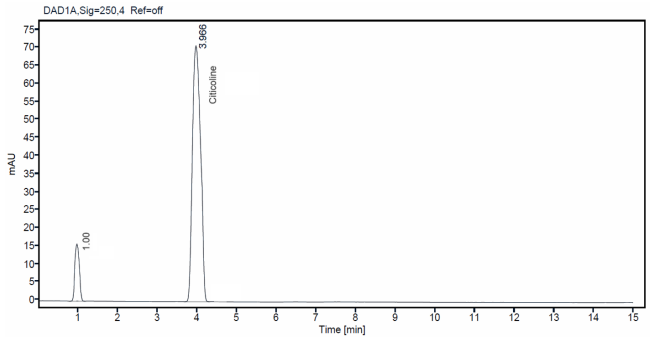



**Figure 4:** Acid degradation

and then 1-mL of 0.1M NaOH was added and made up with water up to the mark. It was then kept in an oven to maintain a temperature of 60ºC for 2 hours. Figure 5 citicoline shows alkali degradation.

*Oxidative degradation*

Hydrogen peroxide ($H_2O_2$) 3% v/v was used for oxidative degradation. About 100 µg/mL of stock solution was transferred into 10 mL of a standard flask, and then 1-mL of ($H_2O_2$) was added and made up with water up to the mark. It was kept at room temperature for 2 hours. Figure 6 citicoline shows oxidative degradation.

*Photolytic degradation*

For photolytic degradation, 100 µg/mL of stock solution was transferred into 10 mL of a standard flask makeup with the desired volume of water up to the mark. Over a period of time nearly 2 hours the drug was subjected to UV light without disturbances, which was then filtered through a 0.45 µm syringe filter. Figure 7 citicoline shows photolytic degradation.

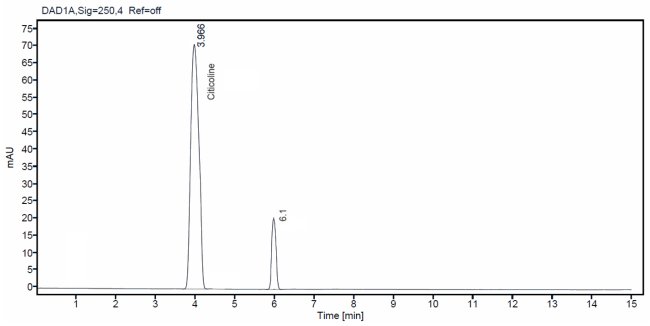



**Figure 5:** Alkali degradation

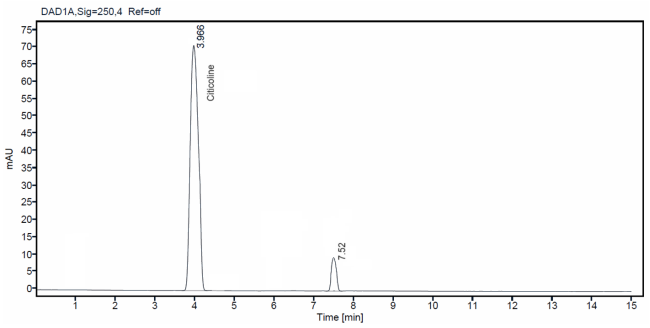



**Figure 6:** Oxidative degradation

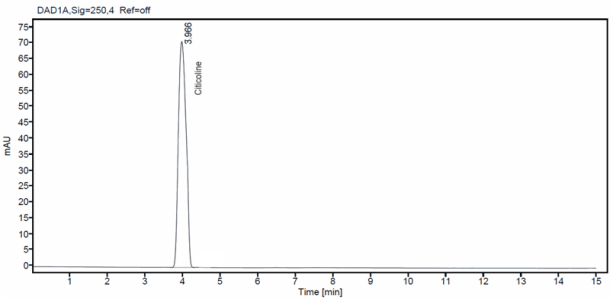



**Figure 7:** Photolytic degradation

*Thermal degradation*

For thermal degradation, 100 µg/mL of stock solution was transferred into 10 mL of a standard flask, makeup with the desired volume of water up to the mark. When the drug was subjected to 60°C for 2 hours which was then filtered through a 0.45 µm syringe filter. Figure 8 citicoline shows thermal degradation and Table 2 shows degradation parameters.

*GC-MS analysis*

An Agilent 6890N gas chromatography (GC) 5975 mass selective detector (MSD) system operating at 70 eV with an ion source temperature set at 230°C was used for GC-MS analysis. A capillary column, i.e., crosslinked 5% phenyl methyl siloxane with 0.33-m-film thickness, was used in the GC. The degradation was performed using a citicoline sample solution, injected in the GC in spitless mode, with the helium carrier gas flow rate set to 1.0 mL/minute.

The GC oven temperature was set at 100°C for 0.5 minutes, then increased to 280°C at 25 minutes and held for 1-minute.

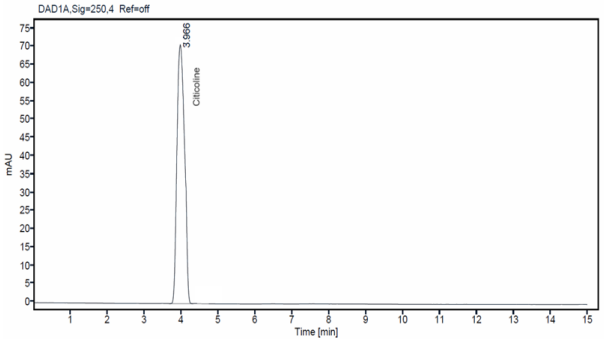



**Figure 8:** Thermal degradation

**Table 2:** Degradation parameters in different conditions

| *S. No* | *Factors* | *Drug recovered in %* | *Drug degradation in %* |
|---|---|---|---|
| 1 | 0.1 M NaOH | 98.65 | 1.35 |
| 2 | 0.1 m HCl | 96.5 | 3.5 |
| 3 | 3% v/v $H_2O_2$ | 99.38 | 0.2 |
| 4 | Photolytic degradation | 95 | 5 |
| 5 | Thermal degradation | 87 | 13 |

The post-run temperature was set to 300°C for 2 minutes to clean out the column before the next injection. Injecting the citicoline sample solution in the GC-MS, a full-scan mass spectrum (MS) of the analyte was typically acquired. The scan range was commonly adjusted to m/z to the expected product's molecular weight, with the maximum number of chemical groups attached. The MSD was employed in the selective ion monitoring mode for quantification. The main purpose of performing the GC-MS was confirmation of the degradation and characterize the degraded products. The identified degraded compound has IUPAC name: 1-hydroxy-1-methoxy-3-(1-Oxidaneyl)-3-propoxydiphospho xanen 1, 3-dioxide. Figure 9 shows degradation in GC- MS.

**Quality by Design**

Quality by Design (QbD) is the approach developed to build the quality of products. The quality cannot be developed by testing it should be developed from the manufacturing process. The primary objective of QbD is to ascertain the intended product quality through the evaluation of variables that have the potential to influence the quality. The ICH guideline Q8 (R2) clearly describes QbD, an approach to method development and validation that commences with pre-established objectives. This approach is rooted in good scientific principles and incorporates quality risk management. AQbD, also known as Analytical Quality by Design, is a comprehensive methodology and developmental strategy that encompasses the entire analytical process, ranging from risk assessment to lifecycle management. Implementing the enhanced (AQbD) approach has been shown to effectively decrease the duration and resources required for developing robust analytical procedures.

**Central Composite Design**

The central composite design is a statistical technique commonly employed in response surface methods to construct a second-order (quadratic) model for the response variable. This design is particularly advantageous since it eliminates the necessity of doing a comprehensive three-level factorial experiment. Coded variables are frequently employed in the construction of this design.

**Method Development and Optimization using AQbD**

Various buffers, such as phosphate, acetate, and formate, were tried at different pH levels. Additionally, multiple solvents were experimented with. Finally, environmentally friendly solvents are chosen to adhere to the principles of green chemistry.

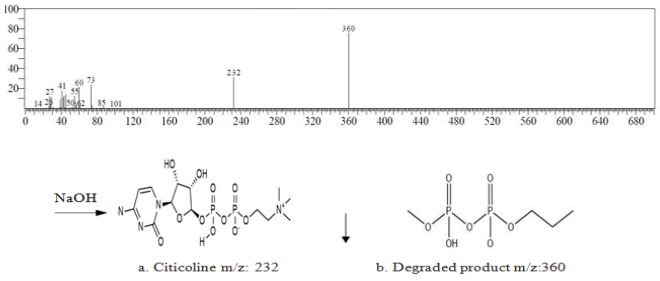



**Figure 9:** Citicoline shows degraded compounds in GC-MS analysis

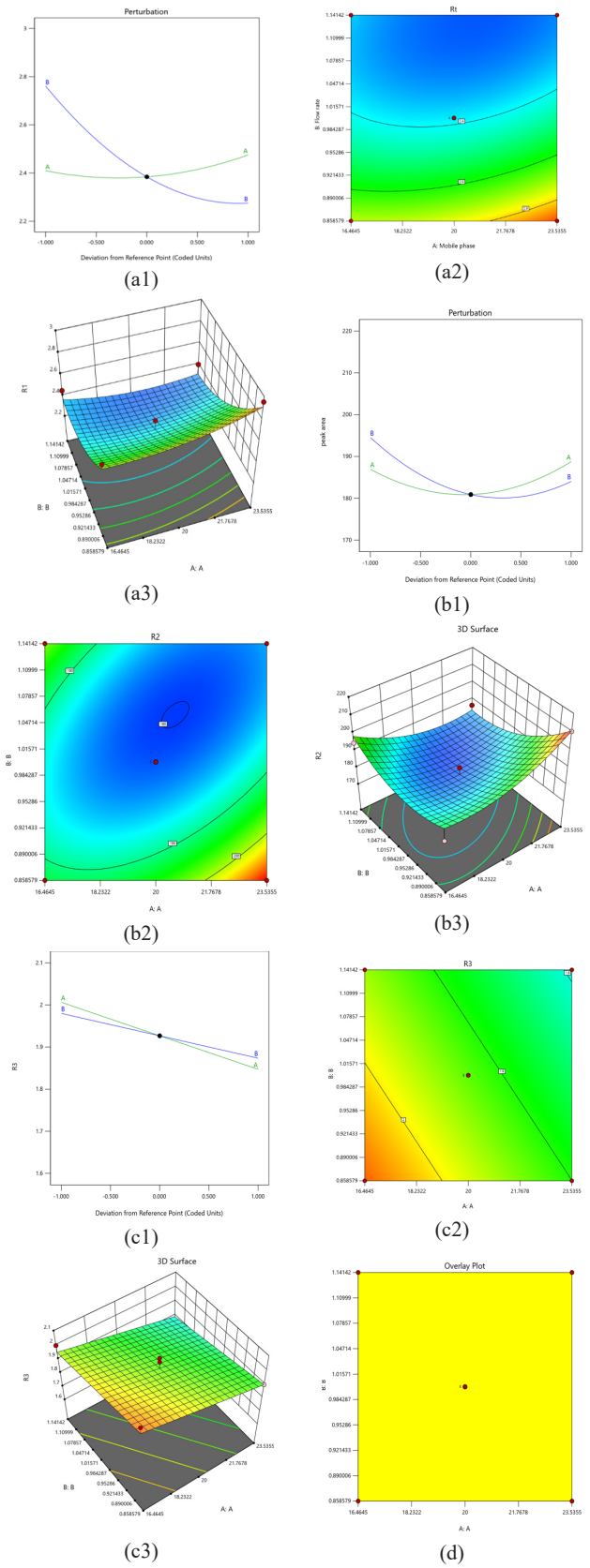



**Figure 10:** a1 – Perturbation plot, a2 – Contour plot, a3 – 3D surface plot of Retention time (Rt), b1 – Perturbation plot, b2 – Contour plot, b3 – 3D surface plot of area, c1 – Perturbation plot, c2 – Contour plot, c3 – 3D surface plot of tailing factor, d – Overlay plot of three factors.

Ethanol was employed in the organic phase, while water was used in the aqueous phase at a ratio of 20:80 v/v. The drug undergoes complete dissolution and subsequent dilution with water without the addition of any buffering reagents. A 0.1% solution of orthophosphoric acid, which also serves as an environmentally acceptable solvent, was used to modify the pH. The method was developed and optimized using the central composite design QbD Design-Expert® Software, specifically version 22.0. Figure: 10 shows Qbd peaks of perturbation, contour, 3 D surface and overlay of 3 factors. The factors are 1; retention time -Rt, 2; area and 3; tailing factor.

## RESULTS

### Stability of the Solutions

The stability of the standard citicoline solution was checked at room temperature for over 72 hours. The %assay value of the standard was compared with the freshly prepared samples.

### Validation Parameters

The most important requirement for drug validation is to establish its appropriateness for its designated use. The proposed methodology was verified by the guidelines specified in ICH Q14.

*Linearity, LoD, LoQ*

A robust linear correlation between the concentration and peak areas. The linearity concentration of citicoline ranges from 10 to 50 µg/mL. The correlation coefficient $R^2$ was 0. 999. The LoD and LoQ values were 5.0098 and 15.18 µg/mL. Figure 11 shows the linearity of citicoline. Table 3 shows validation parameters.

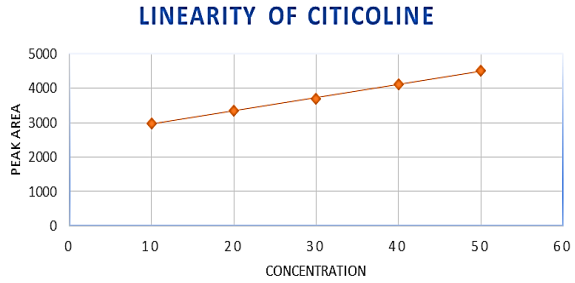



**Figure 11:** Linearity graph of citicoline

**Table 3:** Results of selected validation parameters

| *S. No* | *Parameters* | *Values* |
|---|---|---|
| 1 | Concentration [µg/mL] | 10-50 |
| 2 | Regression equation | y = 38.537x+2571.5 |
| 3 | Correlation coefficient [$R^2$] | 0.999 |
| 4 | Limit of detection [µg/mL] | 5.0098 |
| 5 | Limit of quantification [µg/mL] | 15.18 |
| 6 | Accuracy | 97.9-99.8 |
| 7 | Interday precision | 0.54 |
| 8 | Intraday precision | 0.44 |

### Accuracy and precision

A well-known drug concentration 80, 100, and 120% was analyzed in triplicate with the formulation, and the percentage of recovery was then determined. Great yields were obtained for every concentration. The precision is also known as repeatability. The estimated percentage RSD was less than 2, which shows that the method was very reliable.

## DISCUSSION

The evaluation of the environmental sustainability of the proposed and implemented technique. To demonstrate eco-friendliness, the method was developed on the green solvents following the 12 green chemistry principles guidelines. The proposed method was evaluated using the green evaluation tools.

### Green Analytical Procedure Index

The green analytical procedure index (GAPI) tool is a partially quantitative method that effectively demonstrates a higher representation of images in the shade of red, showing greater toxic effects on the surroundings. Furthermore, the developed method provides clear evidence that it does not exhibit any hazardous indications, which can be attributed to the utilization of ethanol, an environmentally conscious solvent. The proposed method shows a green color pictogram.

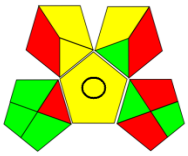
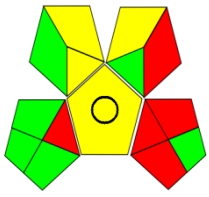
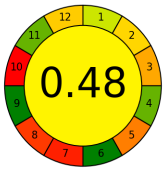
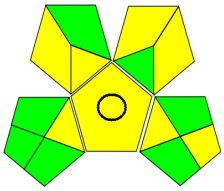
### Analytical Eco-scale

The analytical eco-scale (AES) is proposed as an innovative and comprehensive framework for assessing the environmental sustainability of analytical methodologies. The methodology involves the allocation of penalty points to parameters within an analytical process that deviates from the desired standards of environmentally friendly analysis. This methodology involves the comparison of several metrics and processes within the analytical process. The analytical eco-scale value for the proposed method is 95.

### Analytical Greenness Metric [AGREE]

When evaluating the environmental impact of an analytical approach simple, accurate, precise and sensitive. The drug was resolved, and numerous variables were taken into account, including the quantities and toxicity of reagents, the generation of waste, energy consumption, the number of procedural steps, and considerations related to miniaturization and automation. The utilization of criteria for assessing greenness necessitates the use of specialized tools. The analytical reliability calculator is a proposed evaluation approach that aims to be thorough, adaptable, and clear. It is designed to offer a result that is simply interpretable and instructive. The assessment criteria have been derived from the 12 green analytical chemistry principles and standardized on a single scale ranging from 0 to 1. The concept of significance determines the ultimate score. The outcome is a visual representation, namely a pictogram that displays the ultimate score, the performance of the analytical technique in each criterion, and the weights that the user has allocated. The analytical greenness metric (AGREE) value for the proposed method is 0.84. Table 4 compares the suggested HPLC method *versus* the previously published one in terms of sustainability assessment.

**Table 4:** Comparison of the suggested HPLC method versus the previously published HPLC method in terms of sustainability assessment

| *S. No* | *Reference* | *Conditions* | *Gapi* | *Aes* | *Agree* |
|---|---|---|---|---|---|
| 1 | Shailendra Bindaiya, Ameeta Argal | HPLC: Tetra butyl ammonium hydrogen sulphate buffer: Methanol [95:5] |  | Reagent = 10<br>Instrument = 1<br>Occupational hazard = 0<br>Waste = 0<br>Total = 100-11<br>AES = 89 |  |
| 2 | Sharma O, Chand T | HPLC: potassium di-hydrogen phosphate and Tetra butyl ammonium hydroxide: Methanol [95:5] |  | Reagent=18<br>Instrument =1<br>Occupational hazard=0<br>Waste =0<br>Total =100-19<br>AES = 81 |  |
| 3 | Proposed method | HPLC: Ethanol: Water |  | Reagent=4<br>Instrument =1<br>Occupational hazard=0<br>Waste =0<br>Total =100-5<br>AES = 95 |  |

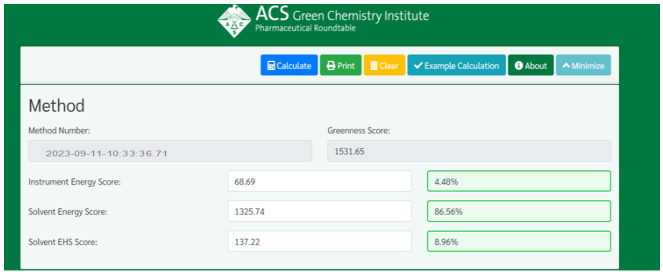



**Figure12:** AMGS calculator

### Analytical Method Greenness Score

One potential approach to examining health, safety, and environmental factors is through the utilization of the high-performance liquid chromatography (HPLC) environmental assessment tool, commonly referred to as analytical method greenness score (AMGS). The AMGS consisted of three distinct categories: instrument energy score, solvent energy score, and solvent environmental health and safety score. The composite score of the method is determined by summing the three individual scores to minimize it and maximize the method's environmental sustainability. The proposed approach yielded a final result of 1531.65, indicating a positive impact of the developed method on the environment. This was achieved by inputting the necessary data into the calculator provided exclusively by the ACS Green Computing Institute to conduct green evaluations. Figure 12 shows the AMGS calculator.

### Carbon Footprint Analysis

The carbon footprint analysis estimates the rise of greenhouse gas emissions in the atmosphere because of the combustion and burning of fossil fuels. Determining the carbon footprint for the developed method is crucial. This study examines the analysis of citicoline using RP-HPLC. The instruments require significantly fewer watts of energy, and the HPLC utilizes approximately 1.5 in the unit of (kWh). This energy consumption results in a substantial reduction in carbon dioxide emissions, thereby yielding minimal environmental impacts. This confirmed that the developed method is greener. The results from the evaluation techniques show the method is green.

## CONCLUSION

This study shows a new way to use AQbD and green analytical chemistry (GAC) in the formulation of citicoline. An experimental design, such as a central composite design, was employed for the statistical optimization investigations. The acquired results determined the optimal operating conditions for the drug within the region of the design space, which were validated through additional trials. Some methods were reported to be harmful to humans and the environment through continuous usage. This is the first method for developing this drug, using only ethanol and water with no buffer. The new procedure adheres to the ICH guidelines and meets the acceptance criteria. Even though this method is precise and effective, it does not adversely affect the environment or human health. In conclusion, the green evaluation techniques revealed that the procedure was the most eco-friendly, completely safe, and readily appropriate for industry and routine monitoring of quality.

## ACKNOWLEDGEMENT

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## REFERENCES

1. Secades JJ, Gareri P. Citicoline: pharmacological and clinical review, 2022 update. Rev Neurol. 2022; 30; 75(s05):S1-S89.
2. De Maagd G, Philip A. Parkinson's Disease and Its Management: Part 1: Disease Entity, Risk Factors, Pathophysiology, Clinical Presentation, and Diagnosis. P & T: a peer-reviewed journal for formulary management. 2015; 40(8):504-532.
3. George DeMaagd, Ashok Philip, Parkinson's disease and Its Management: Part 1: Disease Entity, Risk Factors, Pathophysiology, Clinical Presentation, and Diagnosis. P & T: a peer-reviewed journal for formulary management. 2015; 40(8):504-510,532.
4. Eberhardt R, Birbamer G, Gerstenbrand F, Rainer E, Traegner H. Citicoline in the treatment of Parkinson's disease. Clin Ther. 1990; 12(6):489–95.
5. Que DLS, Jamora RDG. Citicoline as Adjuvant Therapy in Parkinson's disease: A Systematic Review. Clinical Therapeutics. 2021; 43(1):19–31.
6. Wisher D. Martindale the Complete Drug Reference. J Med Libr Assoc. 2012; 100(1):75–76.
7. Jasielski P, Piędel F, Piwek M, Rocka A, Petit V, Rejdak K. Application of Citicoline in Neurological Disorders: A Systematic Review. Nutrients. 2020; 12(10):3113.
8. Ganduri RB, Peddareddigari JR, Dasari NR, Saiempu RK. Stability indicating LC method for the Determination of citicoline sodium in Injection formulation. Int J Pharm Tech Res. 2010; 2:427–33.
9. Patil AS, Pethe A. Quality by Design (QbD): A new concept for development of quality pharmaceuticals. Int J Qual Assur. 2023; 1(4):13–9.
10. Prajapati P, Shah H, Shah, SA. Implementation of QRM and DoE-Based Quality by Design Approach to VEER Chromatography Method for Simultaneous Estimation of Multiple Combined Dosage Forms of Paracetamol. J. Pharm. Innov. 2022; 17:2–18.
11. Monks K, Molnar I, Rieger HJ, Bogati B, Szabo E. Quality by Design: Multidimensional Exploration of the Design Space in High Performance Liquid Chromatography Method Development for Better Robustness Before Validation. J. Chromatogr. A 2012; 1232: 218–230.
12. Sandhu PS, Beg S, Katare OP, Singh B. QbD-Driven Development and Validation of a HPLC Method for Estimation of Tamoxifen Citrate with Improved Performance. J. Chromatogr. Sci. 2016; 54: 1373–1384.
13. Wasim Akram, Navneet Guard. Design expert as a statistical tool for optimization of 5-ASA-loaded biopolymer-based nanoparticles using Box Behnken factorial design. Future journal of pharmaceutical sciences. 2021; 7:146.
14. Shailendra Bindaiya, Ameeta Argal. Development and validation of RP- HPLC method for determination of citicoline monosodium in pharmaceutical preparations. International Journal of

Pharmaceutical Chemistry. 2012; 2(3):85-88.
15. Sharma O, Chand T. Analytical Method Development and Its Validation for Estimation of Citicoline Sodium by Reversed Phase High Performance Liquid Chromatography (RP-HPLC). International Journal of Research in Pharmaceutical and Biomedical Sciences. 2013; 4:550–8.