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ORIGINAL ARTICLE **OPEN ACCESS**

New Analytical Method Development and Validation for Estimation of Citicoline and Methylcobalamin in Bulk and Tablet by RP-HPLC

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ABSTRACT
A novel, precise, accurate and cost-effective isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed for the simultaneous estimation of Citicoline and Methyl cobalamin in bulk and pharmaceutical dosage forms. The separation of the analytes was achieved using a Phenomenex Gemini C18 (4.6mm × 150mm, 5µm) particle size column with a mobile phase composed of triethylamine buffer and methanol in a32:68 v/v ratio flowing at a rate of 1.0 ml/min. Detection was carried out at 248 nm. The linearity range for both Citicoline and Methyl cobalamin was established, with correlation coefficients (r^2) exceeding 0.999. Precision studies demonstrated % RSD values less than 2% for both drugs across all selected concentrations. The proposed method exhibited excellent accuracy, with mean recoveries of 100.1873% and 100.748% for Citicoline and Methyl cobalamin, respectively. The limits of detection (LOD) and quantification (LOQ) were determined as 2.6 µg/ml and 7.8 µg/ml for Citicoline and 3.4 µg/ml and 10.2 µg/ml for Methyl cobalamin, respectively. The validation of the method was conducted in accordance with International Conference on Harmonization (ICH) guidelines, demonstrating its suitability for routine analysis of Citicoline and Methyl cobalamin in pharmaceutical formulations.
Keywords: *Citicoline and Methyl cobalamin, RP-HPLC, ICH Guidelines, Validation*

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INTRODUCTION

Citicoline (CDP-choline) is a naturally occurring choline derivative that plays a crucial role in brain functioning. It serves as a precursor to phosphatidylcholine, a major phospholipid component of cell membranes. Citicoline has demonstrated potential therapeutic benefits, including enhanced cognitive function, reduced neurodegeneration, and protection against stroke (1). Methyl cobalamin (vitamin B12) is an essential nutrient involved in various vital biological processes, including DNA synthesis, nerve cell function, and red blood cell formation. Methyl cobalamin deficiency can lead to a range of neurological and hematological disorders. The simultaneous determination of citicoline and methyl cobalamin in bulk and pharmaceutical dosage forms poses a significant analytical challenge due to their similar chemical structures and properties. While various HPLC methods have been developed for their analysis, most are complex, time-consuming, and require expensive equipment (2). In this study, we present a simple, rapid, and cost-effective RP-HPLC method for the simultaneous determination of citicoline and methyl cobalamin in bulk and pharmaceutical dosage forms. The proposed method utilizes a Phenomenex Gemini C18 column a mobile phase composed of triethylamine buffer and methanol for the separation of Citicoline and Methyl cobalamin. Detection is carried out at 248nm (3).

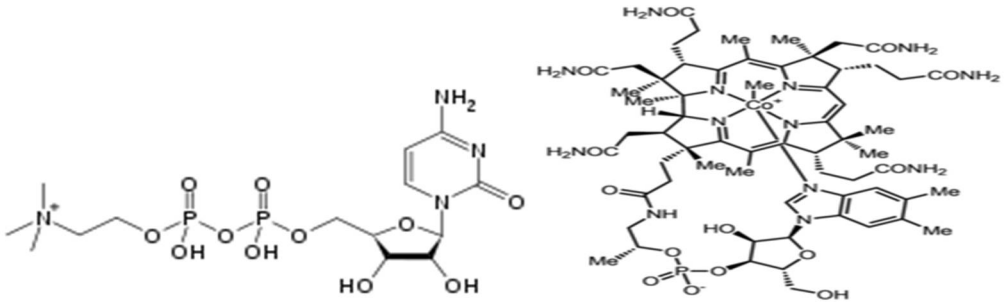

Figure 1. Chemical structure of Citicoline and Methyl cobalamin

The International Conference on Harmonization's (ICH) recommendations were followed when validating the method. Linearity, precision, accuracy, specificity, robustness, limit of detection (LOD), and limit of quantification (LOQ) of the method were thoroughly evaluated. The proposed method was successfully applied to the quantitative analysis of commercially available dosage forms, demonstrating its suitability for routine analysis (4). The development of a simple, rapid and cost-effective RP-HPLC method for the simultaneous determination of Citicoline and Methyl cobalamin in bulk and pharmaceutical dosage forms has significant applications in the pharmaceutical industry and clinical settings. This method can be used for quality control of pharmaceutical formulations containing Citicoline and Methyl cobalamin, ensuring the consistent production of high-quality medications (5). Moreover, it can be employed in clinical studies to monitor the concentrations of citicoline and methyl cobalamin in biological fluids, providing valuable insights into their pharmacokinetics and pharmacodynamics. Furthermore, the proposed method has important social implications. Citicoline and Methyl cobalamin are increasingly being recognized for their potential therapeutic benefits in various neurological disorders, including Alzheimer's disease, Parkinson's disease, and stroke. The accurate and reliable determination of these compounds is crucial for optimizing their dosage regimens and evaluating their clinical efficacy. The availability of a simple, rapid, and cost-effective analytical method can facilitate the wider adoption of Citicoline and Methyl cobalamin in clinical practice, potentially improving the quality of life for individuals suffering from neurological conditions. The development of the RP-HPLC method for the simultaneous determination of Citicoline and Methyl cobalamin represents a significant contribution to the field of analytical chemistry (5,6). The method addresses the limitations of existing methods by offering simplicity, rapidity, and cost-effectiveness. It also demonstrates the applicability of RP-HPLC in the analysis of compounds with similar chemical structures, providing a valuable tool for researchers and analysts in the pharmaceutical and biomedical fields. Literature review suggests that RP- HPL is a reliable and sensitive method for the determination of citicoline and methyl cobalamin in pharmaceutical formulations. Citicoline and methyl cobalamin are susceptible to degradation under acidic, alkaline, and photolytic conditions. Citicoline and methyl cobalamin are used in the treatment of a variety of neurological disorders.

MATERIAL AND METHODS

The HPLC system used in this study consisted of a WATERS Alliance 2695 separation module controlled by Empower 2 software and equipped with a 996 PDA detector. The chemicals used in the experiment were citicoline from Sura labs, methyl cobalamin from Sura labs, water, and methanol for HPLC from LICHROSOLV (MERCK), and acetonitrile for HPLC from Merck. A mobile phase was prepared by mixing 320 mL (32%) of HPLC-grade methanol and 680mL (68%) of TEA buffer. The mixture was degassed in a digital ultrasonicator for 15 minutes to remove dissolved gases and then filtered through a 0.45 µm filter under vacuum filtration to remove any particulate matter. This mobile phase was used as the diluent for sample preparation.

METHOD VALIDATION (7-9)

Preparation of mobile phase

Accurately measured 320ml (32%) of HPLC Methanol and 680ml of TEA buffer (68%) were mixed and degassed in a digital ultra sonicator for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

VALIDATION PARAMETERS

SPECIFICITY

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Citicoline and Methyl cobalamin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.5ml of the above Citicoline and 0.3ml of the Methyl cobalamin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution

Take average weight of the Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight ofCiticoline and Methyl cobalamin sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.5ml of the above Citicoline and 0.3ml of the Methyl cobalamin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \text{Weight of tablet} \times 100 \times \text{Label claim}$$

PREPARATION OF DRUGSOLUTIONS FOR LINEARITY

Accurately weigh and transfer 10 mg of Citicoline and Methyl cobalamin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Procedure

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

PRECISION

Preparation of Citicoline and Methyl cobalamin Product Solution for Precision

Accurately weigh and transfer 10 mg of Citicoline and Methyl cobalamin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.5ml of the above Citicoline and 0.3ml of the Methyl cobalamin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent. The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

ACCURACY

For preparation of 50% Standard stock solution

Accurately weigh and transfer 10 mg of Citicoline and Methyl cobalamin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.25ml of the above Citicoline and 0.15ml of the Methyl cobalamin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution

Accurately weigh and transfer 10 mg of Citicoline and Methyl cobalamin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.5ml of the above Citicoline and 0.3ml of the Methyl cobalamin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution

Accurately weigh and transfer 10 mg of Citicoline and Methyl cobalamin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.75ml of the above Citicoline and 0.45ml of the Methyl cobalamin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Citicoline and Methyl cobalamin and calculate the individual recovery and mean recovery values.

ROBUSTNESS

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution

Accurately weigh and transfer 10 mg of Citicoline and Methyl cobalamin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.5ml of the above Citicoline and 0.3ml of the Methyl cobalamin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of flow conditions

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded

Effect of Variation of mobile phase organic composition

The sample was analyzed by variation of mobile phase i.e. Methanol: TEA buffer pH 4.8 was taken in the ratio and 27:73, 37:63 instead of 32:68, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Method Development and Optimization

A simple, rapid and cost-effective RP-HPLC method was developed for the simultaneous determination of Citicoline and Methyl cobalamin in bulk and pharmaceutical dosage forms. The chromatographic separation was achieved on a Phenomenex Gemini C18 (4.6mm×150mm, 5.0 µm) particle size column using a mobile phase composed of triethylamine buffer and methanol in a 32:68 v/v ratio, at a flow rate of 1.0 ml/min. Detection was carried out at 248 nm. The retention times for citicoline and methyl cobalamin were3.297 min and5.405 min, respectively. The method was optimized by varying the mobile phase composition, flow rate, and column temperature. The optimized conditions were mobile phase ratio of methanol:TEA bufferpH 4.8 (32:68v/v), flow rate of 1ml/min, and column temperature of 38˚C.

Linearity

The linearity of the method was evaluated by constructing calibration curves for both Citicoline and Methyl cobalamin over the concentration range of 30-70µg/ml and 10-50µg/ml, respectively as shown in table 1. The correlation coefficients (R^2) for Citicoline and Methyl cobalamin were found to be 0.9998 and 0.9994, respectively, indicating a good linear relationship between the concentration of the analytes and the peak area.

Table 1: Linearity values of Citicoline

Concentration µg/ml	Average Peak Area
30	545894
40	725985
50	897856
60	1068594
70	1245698

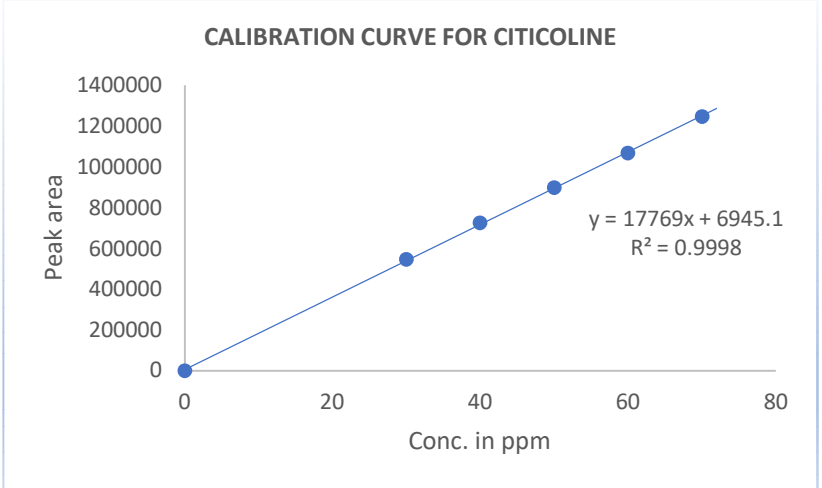

Figure 2. Calibration Curve of Citicoline

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater with Correlation Coefficient (r) is 0.99, and the intercept is 6945. These values meet the validation criteria.

Table 2: Linearity values of Methyl cobalamin

Concentration µg/ml	Average Peak Area
10	2038
20	3859
30	5698
40	7489
50	9218

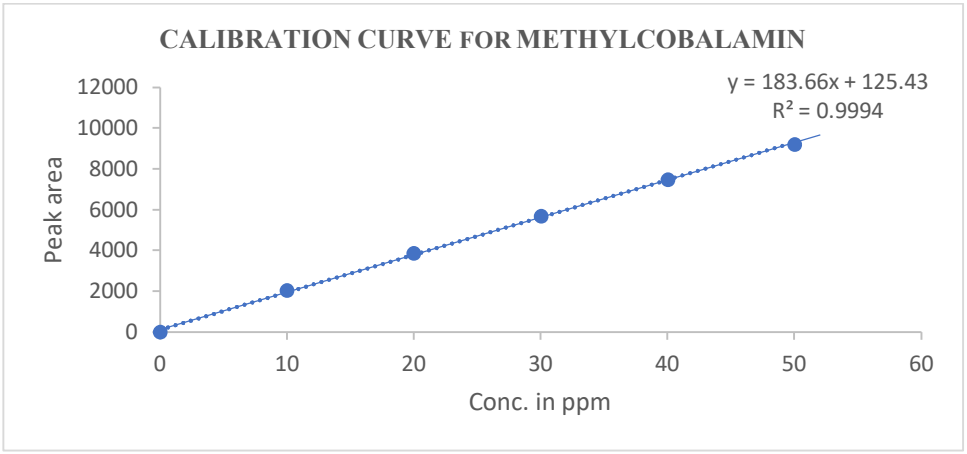

Figure3. Calibration Curve of Methyl cobalamin

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater with correlation Coefficient (r) is 0.99, and the intercept is 125.4. These values meet the validation criteria.

Precision

The precision of the method was determined by performing intra- and inter-day precision studies. The intra-day precision was evaluated by injecting five replicate samples of Citicoline and Methylcobalamin at the concentration level of 30µg/ml and 4.5µg/ml, respectively. The inter-day precision was evaluated by injecting five replicate samples of Citicoline and Methylcobalamin at the concentration level of 30µg/ml and 4.5µg/ml, respectively, on three consecutive days. The % RSD values for both intra and inter-day precision were less than 2%, indicating that the method is precise.

Table 3: Results of Intermediate precision for Citicoline

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Citicoline	3.211	868956	43659	7985	1.26
2	Citicoline	3.211	869857	43985	7954	1.27
3	Citicoline	3.210	865983	43879	7946	1.26
4	Citicoline	3.212	866587	43865	7963	1.27
5	Citicoline	3.211	864256	43875	7964	1.26
6	Citicoline	3.297	868974	43562	7942	1.26
Mean			867435.5			
Std.Dev.			2167.095			
%RSD			0.249828			

Acceptance criteria

- %RSD of six different sample solutions should not more than 2

Table 4: Results of Intermediate precision for Methyl cobalamin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Methyl cobalamin	5.411	5785	3789	6659	1.37
2	Methyl cobalamin	5.410	5798	3758	6625	1.38
3	Methyl cobalamin	5.420	5766	3746	6649	1.38
4	Methyl cobalamin	5.423	5746	3795	6675	1.37
5	Methyl cobalamin	5.419	5782	3761	6653	1.38
6	Methyl cobalamin	5.409	5786	3752	6627	1.37
Mean			5777.167			
Std.Dev.			18.40018			
%RSD			0.318498			

Acceptance Criteria:

- %RSD of six different sample solutions should not more than 2.

Accuracy

The accuracy of the method was determined by performing recovery experiments at three different concentration levels (50%,100%, and150%). The percentage recoveries of Citicoline and Methyl cobalamin were found to be 100.1873% for Citicoline and 100.748% for Methyl cobalamin respectively, indicating that the method is accurate as shown in table 5 and 6.

Table 5: The accuracy results for Citicoline

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	451144.3	25	24.998	99.992%	100.1873%
100%	897248.3	50	50.104	100.208%	
150%	1344562	75	75.278	100.362%	

Acceptance Criteria: The percentage recovery was found to be within the limit (98-102%).
The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table 6: The accuracy Results for Methyl cobalamin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	2895	15	15.084	100.560%	100.748%
100%	5685.333	30	30.282	100.940%	
150%	8449	45	45.335	100.744%	

Acceptance Criteria: The percentage recovery was found to be within the limit (98-102%).
The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Specificity

The specificity of the method was confirmed by the absence of interfering peaks from other components in the sample matrix. The chromatograms obtained from the analysis of standard and sample solutions showed no interfering peaks at the retention times of Citicoline and Methyl cobalamin.

Table 7: Peak Results for Assay sample of Citicoline

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Citicoline	3.297	865985	43659	1.26	7985
2	Citicoline	3.294	865798	43875	1.26	7925
3	Citicoline	3.295	865456	43659	1.27	7946

Table 8: Peak Results for Assay sample of Methyl cobalamin

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Methyl cobalamin	5.435	5789	3659	1.37	6659	6.9
2	Methyl cobalamin	5.417	5798	3684	1.38	6689	7.0
3	Methyl cobalamin	5.434	5749	3695	1.38	6648	6.9

The % purity of Citicoline and Methyl cobalamin in pharmaceutical dosage form was found to be 99.82%.

Robustness

The robustness of the method was evaluated by varying the flow rate and mobile phase composition. The flow rate varies from 0.9ml/min to 1.1ml/min, and the mobile phase composition was varied by ±5%. The results showed that the method was not significantly affected by these changes.

Table 9: Results for Robustness- Citicoline

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0mL/min	859856	3.297	7896	1.24
Less Flow rate of 0.9mL/min	915847	3.639	7251	1.20
More Flow rate of 1.1mL/min	842564	2.859	7415	1.21
Less organic phase (about 5 % decrease in organic phase)	825498	3.460	7365	1.23
More organic phase (about 5 % Increase in organic phase)	814578	3.022	7258	1.22

Acceptance Criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Table 10: Results for Robustness Methyl cobalamin

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1mL/min	5698	5.405	6582	1.36
Less Flow rate of 0.9mL/min	6452	6.250	6785	1.32
More Flow rate of 0.8mL/min	5254	4.863	6365	1.34
Less organic phase (about 5 % decrease in organic phase)	5487	6.196	6254	1.38
More organic phase (about 5 % Increase in organic phase)	5369	5.010	6298	1.33

Acceptance Criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION

The developed Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method represents a robust, reliable, and efficient analytical approach for the simultaneous quantification of citicoline and methylcobalamin in pharmaceutical formulations. This method has been thoroughly validated and demonstrates key attributes such as simplicity, accuracy, precision, and linearity, ensuring its effectiveness for routine quality control in pharmaceutical analysis. The RP-HPLC method provides reliable separation and quantification of both active pharmaceutical ingredients (APIs), with the ability to resolve them from potential interfering substances, ensuring high specificity. The accuracy of the method has been verified through recovery studies, confirming its suitability for precise determination of citicoline and methylcobalamin at various concentrations within the specified range. Additionally, the method exhibits excellent precision, with low intra-day and inter-day variation, making it highly reproducible across different testing conditions. The linearity of the method has been established over a broad concentration range, ensuring reliable measurement of both compounds in pharmaceutical products. This positions the RP-HPLC method as an ideal analytical tool for routine quality control and stability testing of citicoline and methylcobalamin-containing formulations.

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