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Development and Validation of RP- HPLC method for determination of Citicoline and Methycobalamin in bulk and its Pharmaceutical Dosage form

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ABSTRACT: Background: A new, simple, accurate, rugged, robust and precise RP-HPLC method for the simultaneous determination of Citicoline and Methylcobalamin in bulk and its pharmaceutical dosage form has been developed and validated as per ICH Guidelines. **Aim:** The research was aimed to develop and validate analytical method for the simultaneous determination of Citicoline and Methylcobalamin in bulk and its pharmaceutical dosage form by RP-HPLC. **Methods:** HPLC of waters with Kromasil ODS C18 (4.6 × 250 mm) 5 µm column was used for chromatographic separation. The waters injector and PDA Detector was used in chromatographic study. The mobile phase of methanol: acetonitrile (40: 60 v/v) was used in the study at flow rate of 1ml/min. Wavelength selected for detection was 274 nm and injection volume was 10µl. **Results:** By using the developed method, retention time of Citicoline and Methylcobalamin was found to be 1.862 and 2.490 min respectively. The method has been validated for linearity, accuracy and precision. Linearity of Citicoline and Methylcobalamin were in the range of 60 to 140 and 40 to 120µg/ml respectively. The percentage recoveries obtained for Citicoline and Methylcobalamin were found to be in range of 98.0 to 102.0 %. LOD and LOQ were found to be 2.1 and 6.3 µg/ml for Citicoline, whereas 1.6 and 4.8 µg/ml for Methylcobalamin. **Conclusion:** From the present study it can be concluded that the proposed method is simple, sensitive, precise, specific, accurate and reproducible. Results of validation parameters demonstrated that the analytical procedure is suitable for its intended purpose and meets the criteria defined in ICH Q2R1.

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INTRODUCTION:

High Pressure Liquid Chromatography was developed in the mid-1970 and was improved with the development of column packing material and the additional convenience of on-line detectors [1,2]. The various components of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or automatic), guard column, analytical columns, detectors, recorders and/or integrators. Citicoline has a very low toxicity

profile in animals and humans. Clinically, doses of 2000 mg per day have been observed and approved. Minor transient adverse effects are rare and most commonly include stomach pain and diarrhea [3,4]. Citicoline is available as a supplement online and in stores. It is sold in over 70 countries under a variety of brand names that areCebroton, Ceraxon, Cidilin, Citifar, Cognizin, Difosfocin, Hipercol, NeurAxon, Nicholin, Sinkron,Somazina, Synapsine, Startonyl and Trausan [5,6]. The main aim of the present study is to develop of an accurate, precise, sensitive, selective, reproducible and rapid analytical technique for cost effective simultaneous determination of Citicoline and Methylcobalamin in bulk its pharmaceutical dosage form.

MATERIALS AND METHODS:

Materials:

The methanol and acetonitrile was purchased from S.D. Fine chemical, Mumbai. Citicoline and Methylcobalamin tablets were purchased from local market of Moinabad. Citicoline and Methylcobalamin pure drug was procured as gift sample from Pharmaceutical Company. All other chemicals and reagents used in this study are of analytical grade and were procured from an authorized dealer.

Table 1. HPLC method development protocol.

Parameters	Specified data
Instrument used	Waters HPLC with auto sampler and PDA detector 996 model
Temperature	Ambient
Column	Kromasil ODS C18 (4.6 × 250 mm) 5 µm
Mobile phase	Methanol: Acetonitrile (40: 60 v/v)
Flow rate	1 ml/ min
Wavelength	274 nm
Inj. volume	10 µl
Run time	4 min

Preparation of Mobile Phase:

Accurately 400 ml (40 %) of Methanol and 600 ml of Acetonitrile (60 %) were measured and mixed. The mixture was degassed in digital ultra sonicator (Ultrasonicator, Elma Elmasonic E Plus EP30H, USA) for 15 min and then the solution was filtered through 0.45 µ filter under vacuum filtration [7].

Diluent Preparation:

The Mobile phase was used as the diluent.

Method validation parameters [8-10]:

***System suitability*:**

Accurately 10 mg of Citicoline and Methylcobalamin working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To this flask, 7 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution). Further 1 ml of Citicoline and 0.8 ml of Methylcobalamin was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents.

***Procedure*:**

The standard solution was injected for five times and the area for all five injections in HPLC was measured. The % RSD for the area of five replicate injections was found to be within the specified limits.

Specificity study of drug [11,12]:

***Preparation of Standard Solution*:**

Accurately 10 mg of Citicoline and Methylcobalamin working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To this flask, 7 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution). Further 1 ml of Citicoline and 0.8 ml of Methylcobalamin was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents.

***Preparation of Sample Solution*:**

The average weight of ten Tablets was taken and crushed in a mortar by using pestle. The powder weight equivalent to 10 mg of Citicoline and Methylcobalamin sample was taken into a 10 ml clean dry volumetric flask. To the flask about 7 ml of diluent was added and sonicated to dissolve it completely and the volume was made up to the mark with the same solvent. Further 1 ml of Citicoline and 0.8 ml of Methylcobalamin was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents.

***Procedure*:**

The three replicate injections of standard and sample solutions were injected and calculate the assay by using formula:

$$\text{Assay (\%)} = \frac{(SA \times WS \times DS \times P \times WT)}{(StA \times DOS \times WOS \times LC)} \times 100 \quad \text{.....(1)}$$

Where SA and StA are sample and standard area. WS and DOS are weight and dilution of standard. P is purity.

WT and WOS are weight of tablet and of sample. LC is label claim.

Precision (Repeatability) [13,14]:

***Preparation of Citicoline and Methylcobalamin Product Solution for Precision*:**

Accurately 10 mg of Citicoline and Methylcobalamin working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To this flask, 7 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution). Further 1 ml of Citicoline and 0.8 ml of Methylcobalamin was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents. The standard solution was injected for five times and areas are measured for all five injections in HPLC. The % RSD for the area of five replicate injections was determined, which is to be within the specified limits.

Intermediate precision [15]:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

***Procedure*:**

Day 1: The standard solution was injected for five times and the area was measured for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

Day 2: The standard solution was injected for five times and the area was measured for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

Accuracy [16]:

***Preparation of Standard stock solution*:**

Accurately 10 mg of Citicoline and Methylcobalamin working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To this flask, 10 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution of strength 1000 µg/ml). Further 1 ml of Citicoline and 0.8 ml of Methylcobalamin was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents.

***For preparation of 50 % Standard stock solution*:**

Accurately 10 mg of Citicoline and Methylcobalamin working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To this flask, 7 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution of strength 1000 µg/ml). Further 1 ml of Citicoline and 0.8 ml of Methylcobalamin was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents.

For preparation of 100% Standard stock solution:

Accurately 10 mg of Citicoline and Methylcobalamin working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To this flask, 10 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution). Further 0.4 ml of Citicoline and 0.5 ml of Methylcobalamin was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents.

***For preparation of 150 % Standard stock solution*:**

Accurately 10 mg of Citicoline and Methylcobalamin working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To this flask, 7 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution). Further 1.5 ml of Citicoline and 1.2 ml of Methylcobalamin was pipetted from the above stock solutions into a 10 ml volumetric flask and it was diluted up to the mark with diluents.

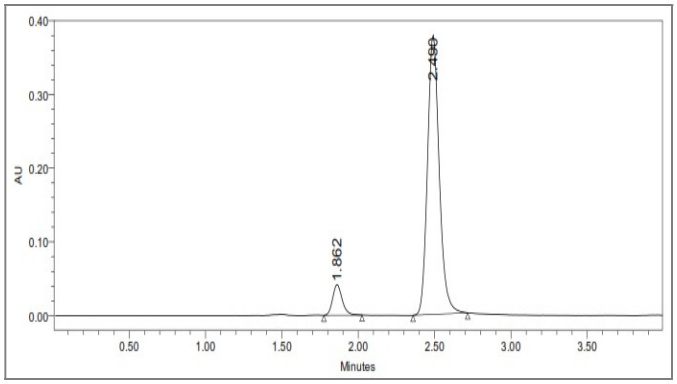

Fig1. The optimized chromatographic data of Citicoline and Methylcobalamin.

***Procedure*:**

About three replicate injections of individual concentrations (50, 100 and 150 %) were injected under the optimized conditions. The chromatograms were recorded and the peak responses were measured. The amount of Citicoline and Methylcobalamin found out

and added was calculated. The individual recovery and mean recovery values were calculated.

Preparation of drug solutions for linearity [17]:
Accurately 10 mg of Citicoline and Methylcobalamin working standard were weighed and transferred into a 10 ml of clean dry volumetric flasks. To this flask, 7 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent (Stock solution).

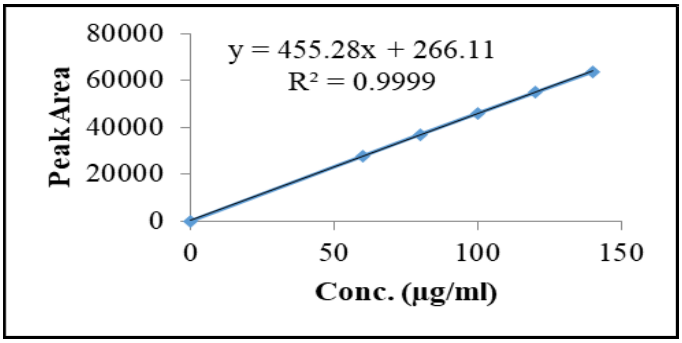

Fig 2. Calibration graph for Citicoline.

***Preparation of Level – I (60 µg/ml of Citicoline & 40 µg/ml of Methylcobalamin)*:**
About 0.6 ml of Citicoline and 0.4 ml of Methylcobalamin was pipetted out from stock solutions. It was taken in a 10 ml of volumetric flask and diluted up to the mark with diluent.

***Preparation of Level – II (80 µg/ml of Citicoline & 60 µg/ml of Methylcobalamin)*:**
About 0.8 ml of Citicoline and 0.6 ml of Methylcobalamin was pipetted out from stock solutions. It was taken in a 10 ml of volumetric flask and diluted up to the mark with diluent.

***Preparation of Level – III (100 µg/ml of Citicoline & 80 µg/ml of Methylcobalamin)*:**
Pipette out 1ml of Citicoline and 0.8ml of Methylcobalamin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

***Preparation of Level – IV (120 µg/ml of Citicoline & 100 µg/ml of Methylcobalamin)*:**
About 1.2 ml of Citicoline and 1 ml of Methylcobalamin was pipetted out from stock solutions. It was taken in a 10 ml of volumetric flask and diluted up to the mark with diluent.

***Preparation of Level – V (140* µg/ml *of Citicoline & 120 µg/ml of Methylcobalamin)*:**
About 1.4 ml of Citicoline and 1.2 ml of Methylcobalamin was pipetted out from stock solutions. It was taken in a 10 ml of volumetric flask and diluted up to the mark with diluent.

***Chromatographic Procedure*:**
Each level of solution was into the chromatographic system and the peak area was measured. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated.

Robustness [18]:
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 10 mg of Citicoline and 10 mg of Methylcobalamin working standard was weighed and transferred into a 10 ml of clean dry volumetric flasks to which about 7 ml of diluents was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent. (Stock solution). Further 1.0 ml of Citicoline and 0.8 ml of Methylcobalamin from the above stock solutions was pipetted into a 10 ml volumetric flask and diluted up to the mark with diluents.

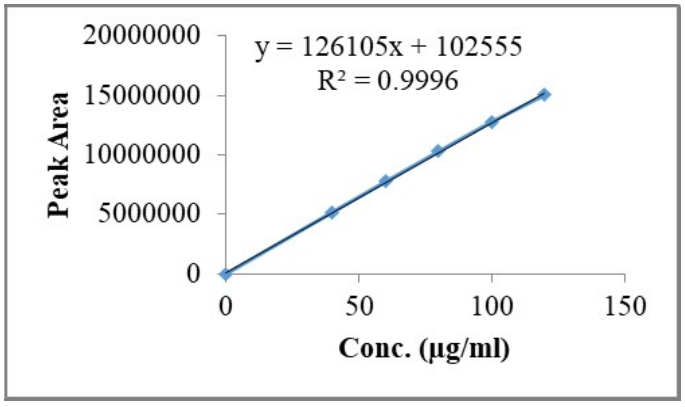

Fig 3. Calibration curve for Methylcobalamin.

***Effect of Variation of flow conditions*:**
The sample was analyzed at solvent flow rate of 0.9 and 1.1 ml/min instead of 1ml/min, remaining conditions are same. About 10 µl of the above sample was injected twice and chromatograms were recorded.

***Effect of Variation of mobile phase organic composition*:**
The sample was analyzed by variation of mobile phase i.e. Methanol: Acetonitrile (40: 60 v/v) was taken in the ratio and 45: 55, 35: 65 instead 40: 60, remaining conditions are same. About 10 µl of the above sample was injected twice and chromatograms were recorded.

Table 2. The chromatographic study parameters data of Citicoline and Methylcobalamin.

Sl. No.	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Citicoline	1.862	46582	3528	--	1.48	6854
2	Methylcobalamin	2.490	8584685	65259	6.82	1.25	8962

Table 3. The system suitability parameters data for Citicoline and Methylcobalamin.

Sl. No.	Name	Retention time (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Citicoline	1.874	45895	3586	--	1.49	6895
2	Methylcobalamin	2.501	8585698	65856	6.83	1.25	8924

Table 4. The Linearity data of Citicoline.

Sl. No.	Linearity Level	Concentration (µg/ml)	Area
1	I	60	27758
2	II	80	36965
3	III	100	45982
4	IV	120	54859
5	V	140	63674
Correlation Coefficient			0.999

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

Table 5. Linearity data of Methylcobalamin.

Sl. No.	Linearity Level	Concentration(µg/ml)	Area
1	I	40	5175985
2	II	60	7746858
3	III	80	10284685
4	IV	100	12789854
5	V	120	15059854
Correlation Coefficient			0.999

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

RESULTS AND DISCUSSION:

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Citicoline and Methylcobalamin was done by RP-HPLC. The mobile phase was optimized with consists of methanol: acetonitrile (40: 60 v/v) mixed in the ratio of 40: 60 % v/ v. A Kromasil ODS C18 (4.6 × 250 mm) 5 µm or equivalent chemically bonded to porous silica particles was used as stationary phase. The solutions were chromatographed at a constant flow rate of 1 ml/min [19-21]. From the above chromatogram (Table 2 and Fig 1), it was observed that the Citicoline and Methylcobalamin peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial. The retention time of Citicoline and Methylcobalamin was found to be 1.862 and 2.49 min respectively. The linearity range of Citicoline and Methylcobalamin were found to be from 60 to 140 µg/ml, 40 to 120 µg/ml respectively. Linear regression coefficient for both the drugs was not more than 0.999. The values of % RSD are less than 2 % indicating accuracy and precision of the method. The percentage recovery varies from 98.0 to 102.0 % of Citicoline and Methylcobalamin. LOD and LOQ were found to be within limit.

CONCLUSION:

The results obtained on the validation parameters met ICH requirements. The method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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