



ANALYTICAL RP-HPLC METHOD FOR DEVELOPMENT AND VALIDATION OF CITICOLINE SODIUM AND METHYLCOBALAMIN IN COMBINED TABLET FORMULATION

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Abstract:

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method have been developed and validated for the estimation of Citicoline Sodium and Methylcobalamin in Bulk drug and Pharmaceutical dosage form. The developed method is rapid, accurate, precise, simple and economical than the previous methods. The separation was carried out using Phenomenex Luna, C18, column having 250mm X 4.6mm, 5 μ m particle size, in isocratic mode, with mobile phase containing Acetonitrile: 0.02M KH₂PO₄ [60: 40, v/v]. The flow rate is 1 ml / min and effluents are monitored at 554 nm. Chromatogram showed peak at a retention time of 3.8 min for Citicoline sodium and 2.3 min for Methylcobalamin. The method is validated for system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, LOD and LOQ. Recovery of Citicoline Sodium and Methylcobalamin is found to be in the range of 99.76 - 101.79 % and 110.92% and 111.79%, respectively. The LOD and LOQ for estimation of Citicoline Sodium and Methylcobalamin are found to be 0.003 μ g / ml, 0.001 μ g / ml, and 0.01 μ g / ml, 0.05 μ g / ml respectively. Proposed method can be successfully applied for the quantitative determination of Citicoline Sodium and Methylcobalamin in Bulk drug and Pharmaceutical dosage form.

Keywords: Citicoline (CITI), Methylcobalamin (MCA), RP-HPLC, Method Development, Validation.

Introduction

Citicoline is an intermediate in the generation of phosphatidyl choline from choline. It is chemically 5'-O [hydroxyl ({hydroxyl [2(trimethylammonio) ethoxy] phosphoryl}oxy) phosphoryl] cytidine. Citicoline is a white or offwhite amorphous, hygroscopic powder having molecular weight 488.3g/mol. It helps to improve focus and mental energy and may possibly be useful in the treatment of attention deficit disorder^[1]. Methylcobalamin (MC) is a coenzyme of Vitamin B12 which is biologically active^[2]. The importance of the combination of citicoline and methylcobalamin known to health care system to boost memory, production of brain energy, sustain cognitive function and motor skill and enhance focus and decision making

thought processes. The major indication of the combination is diabetic neuropathy, diabetic gastropathy, memory loss, brain injury, anorexia, nerve rejuvenation, nerve regeneration and alertness^[3]. Several methods were cited in literature for determination of CITI and MCA individually or in combination with another drug by UV-Vis spectroscopy, HPLC, LC-MS and HPTLC but for combination only UV Spectrophotometric method was reported. Hence there is a need to develop a simple, economic, precise and accurate high performance thin layer chromatographic method for Citicoline Sodium in the presence of methylcobalamin in tablet dosage form.

MATERIALS AND METHODS

Instrument:

High performance liquid chromatography (Perkin Elmer USA Series 200) were used for all measurements. Electronic balance (Shimadzu AX-200, Japan) was used for weighing the samples. Double distilled water and Whatmann filter paper (no.41) were used throughout the experimental work.

Materials:

Multicomponent tablet Storax plus (500mg CITI and 0.75mg MCA) manufactured by Intas Pharmaceutical Ltd. All chemicals and reagents used were of AR grade.

Reagents:

All the chemicals used were of AR grade.

METHODS

Preparation of Mobile Phase:

Buffer 0.02 M KH_2PO_4 was prepared by weighing 2.64 g of KH_2PO_4 and dissolving in 1000 ml of water. Mobile phase was prepared by mixing 400 ml of 0.02M KH_2PO_4 Buffer and 600 ml of acetonitrile. The pH was adjusted to 6 using o-phosphoric acid (1%) or triethyl amine (1%) of the mobile phase was 6. Solution was filtered through Whatman filter paper No. 41 and sonicated for 10 min and this solution was used as a mobile phase.

Preparation of Standard Stock Solutions:

CITI (10 mg) and MCA (10 mg) were accurately weighed and transferred to two separate 10 ml volumetric flask and dissolved in few ml of acetonitrile. Volumes were made up to the mark with acetonitrile to yield a solution containing 1000 $\mu\text{g}/\text{ml}$ of CITI and 1000 $\mu\text{g}/\text{ml}$ of MCA, respectively. Appropriate aliquot from above solutions were taken and diluted with mobile phase to obtain final concentration of 100 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ of CITI and MCA respectively.

Selection of Analytical wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. Overlay UV spectra of both the drugs showed that CITI and MCA absorbed appreciably at 254 nm, so detection was carried out at 254 nm (Figure 1).

Optimization of Mobile Phase

The objective of the method development was to resolve chromatographic peaks for active drug ingredients. Various mixtures containing methanol and water, methanol and various buffer, acetonitrile and water, acetonitrile and various buffers were tried as mobile phases in the initial stage of method development such as shown in table 1.

Chromatographic conditions:

Phenomenex Luna C18 column (250 x 4.6 mm id, 5 μm particle size) chromatographic column equilibrated with mobile phase 0.02M KH_2PO_4 buffer: acetonitrile (40:60, v/v) was used. Mobile phase flow rate was maintained at 1 ml min^{-1} and effluents were monitored at 254 nm. The sample was injected using a 20 μL fixed loop, and the total run time was 6 min.

CALIBRATION CURVE FOR CITI AND MCA

Appropriate aliquot of stock solution of CITI and MCA was taken in same 10 ml volumetric flasks. The volume was made up to the mark with mobile phase to obtain final concentration of 0.01, 0.1, 0.5, 1, 5, 10 $\mu\text{g}/\text{ml}$ of CITI and 0.05, 0.1, 0.5, 1, 5, and 10 $\mu\text{g}/\text{ml}$ of MCA, respectively. (Figure 3 & 4).

METHOD VALIDATION^[5,6]

The method was validated for accuracy, precision, linearity, detection limit, quantitation limit and robustness.

1. Linearity

Appropriate aliquots of CITI and MCA working standard solutions were taken in different 10 ml volumetric flasks and diluted up to the mark with mobile phase to obtain final concentrations of 0.01, 0.1, 0.5, 1, 5, 10 $\mu\text{g}/\text{ml}$ of CITI and 0.05, 0.1, 0.5, 1, 5, and 10 $\mu\text{g}/\text{ml}$ of MCA, respectively.

The solutions were injected using a 20 μL fixed loop system and chromatograms were recorded. Calibration curves were constructed by plotting average peak area versus concentrations and regression equations were computed for both the drugs ($n=6$). (Figure 3 & 4).

2. Precision

The repeatability studies were carried out by estimating response of CITI (0.1 $\mu\text{g}/\text{ml}$) and MCA (0.5 $\mu\text{g}/\text{ml}$) six times and results are reported in terms of relative standard deviation. The intra-day and inter-day precision studies were carried out by estimating the corresponding responses 3 times on the same day and on 3 different days for three different concentrations of CITI (0.01, 0.5, 10 $\mu\text{g}/\text{ml}$) and MCA (0.05, 1, 10 $\mu\text{g}/\text{ml}$), and the results are reported in terms of relative standard deviation. The %RSD is within 2.0 indicating the method is precise. (Table 5).

3. Accuracy

The accuracy of the method was determined by calculating recoveries of CITI and MCA by method of standard additions. Known amount of CITI (0, 0.5, 1, 1.5 $\mu\text{g}/\text{ml}$) and MCA (0, 0.5, 1, 1.5 $\mu\text{g}/\text{ml}$) were added to a pre quantified (1 $\mu\text{g}/\text{ml}$ and 1 $\mu\text{g}/\text{ml}$ of CITI and MCA, respectively) sample solution. The amount of CITI and

MCA were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve. The %RSD was found to be within 2.0 indicating the method is accurate. (Table 2).

4.LOD and LOQ

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using following equation as per ICH guidelines.

$$\text{LOD} = 3.3 \times \sigma / S;$$

$$\text{LOQ} = 10 \times \sigma / S;$$

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve. (Table 5).

5.Robustness

Robustness of the method was studied by deliberately changing the experimental conditions like pH of mobile phase and mobile phase ratio. Robustness of the method was determined in triplicate at a concentration level of 0.5 $\mu\text{g}/\text{ml}$ and 0.5 $\mu\text{g}/\text{ml}$ of CITI and MCA respectively. The mean and % RSD of peak areas were calculated. The %RSD was found to be within 2.0 indicating the method is robust. (table 3)

6.Specificity

The specificity of the method was ascertained by analyzing CITI and MCA in presence of excipients like Starch (50%), Lactose (25%), and Magnesium stearate (1.25%) were used for preparation of tablet formulations. Interference due to excipients was noted and amount of drug recovered were calculated. (Table 5).

7.Solution stability

Stability of sample solutions were studied at ambient temperature for 24 h. The solution was found to be Stable.

8.System suitability

A system suitability test was an integral part of the method development to verify that the system is adequate for the analysis of CITI and MCA to be performed. Retention time (Rt), asymmetry factor, resolution and theoretical plates were determined. (Table 4).

ANALYSIS OF MARKETED FORMULATION:

Twenty tablets were weighed and finely powdered. Tablet powder equivalent to 22 mg CITI (and 0.33 mg

MCA) was weighed and transferred in to a 10 ml volumetric flask containing few ml of mobile phase. The flask was sonicated for 5 minutes. The solution was filtered through Whatman filter paper No. 41 in another 10 ml volumetric flask and volume was made up to the mark using mobile phase(STOCK A). Estimation of MCA was carried out from stock A at 254 nm. From the stock A 1 ml aliquot was pipette out in 10 ml volumetric flask and make up the volume with mobile phase(STOCK B).From the stock B 1 ml aliquot was pipette out in 10 ml volumetric flask and make up the volume with mobile phase to obtain the 4.4 $\mu\text{g}/\text{ml}$ of CITI. Estimation of CITI was carried out at 254 nm. (Table 6).

STORAX PLUS[®] manufactured by Intas Pharmaceutical Ltd. Containing 0.75 mg of Methylcobalamin and 500 mg Citicoline Sodium. Overages of Methylcobalamin is added.

RESULT AND DISCUSSION

Linearity was observed in the concentration range of 0.01-10 $\mu\text{g}/\text{ml}$ for Citicoline sodium and 0.05-10 $\mu\text{g}/\text{ml}$ for Methylcobalamin. Marketed brand of tablet (Storax plus) was analyzed and amount of drug determined by proposed method was found to be 101.15% for CITI and 111.22% for MCA as shown in Table 6. The proposed method was validated as per ICH guideline. The accuracy of method was determined at 50, 100 and 150 % level. The percent recovery ranges from 100.56% to 103.20% for CITI and 109.18% to 111.91% for MCA as shown in Table 2. Precision was calculated as interday and intraday variations (% RSD found to be less than 2 for both drugs. From the interday and intraday studies it is supposed that the drug in solution state is stable for a period of 24hour. The proposed method was found to be simple, accurate and rapid for the routine determination of Citicoline sodium and Methylcobalamin in tablet formulation. This method can be successfully used for simultaneous estimation of Citicoline sodium and Methylcobalamin in combined dosage.

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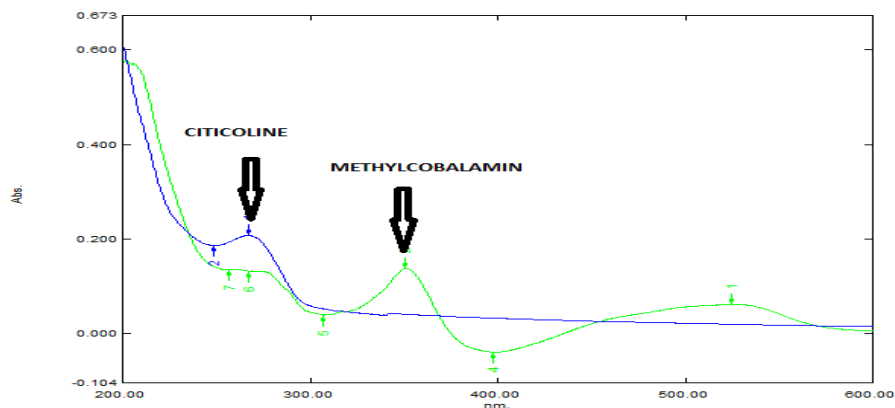


Figure 1: Overlay Spectra Of 10ppm Solutions Of Citicoline Sodium And Methylcobalamin

Table 1: Optimazation of Mobile Phase

MOBILE PHASE	OBSERVATION
Methanol: Water (60:40)	CITI and MCA peaks merge with each other
0.02 M KH_2PO_4 : Methanol (10:90) pH(6.5)	CITI and MCA eluted at same R_t
0.02 M KH_2PO_4 : Methanol (40:60) pH(6.5)	CITI and MCA eluted at same R_t
Acetonitrile: Water (60:40) pH(5.5)	Both the peak were well resolved but R_t was near
Acetonitrile: 0.02 M KH_2PO_4 (90:10) pH(6.5)	Both the peak were well resolved but R_t was near
Acetonitrile: 0.02 M KH_2PO_4 (60:40) pH(6.5)	CITI peak comes at 3.8 min and MCA peak comes at 2.3 min

The mobile phase acetonitrile: 0.02 M KH_2PO_4 (60:40 v/v) pH(6.5) was found to be satisfactory and gave two symmetric and well-resolved peaks for CITI and MCA (Figure 2).

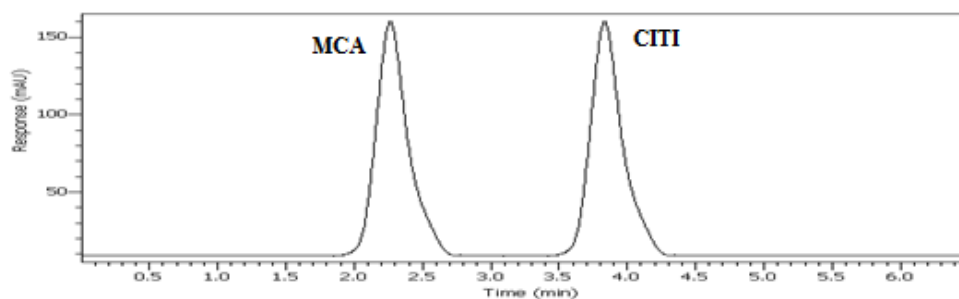


Figure 2: Acetonitrile: 0.02 M KH_2PO_4 (60:40% v/v) (Ph: 6.5)

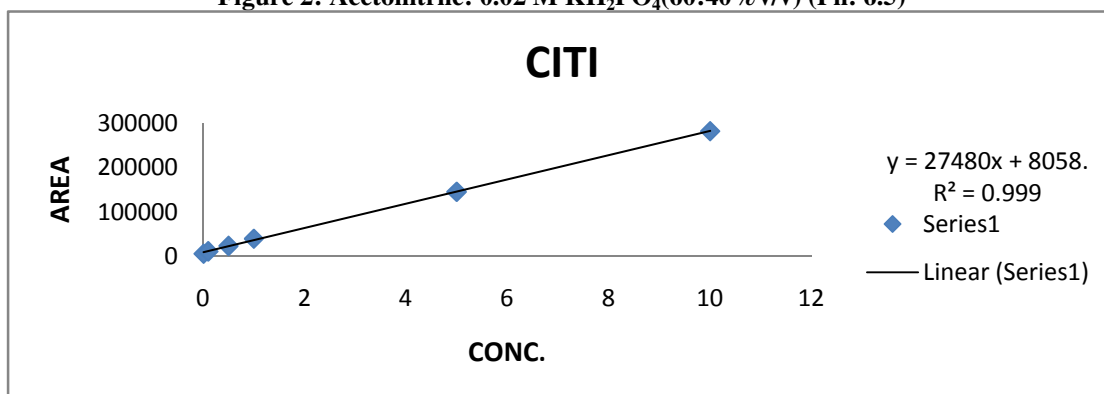


Figure 3: Calibration curve for CITI

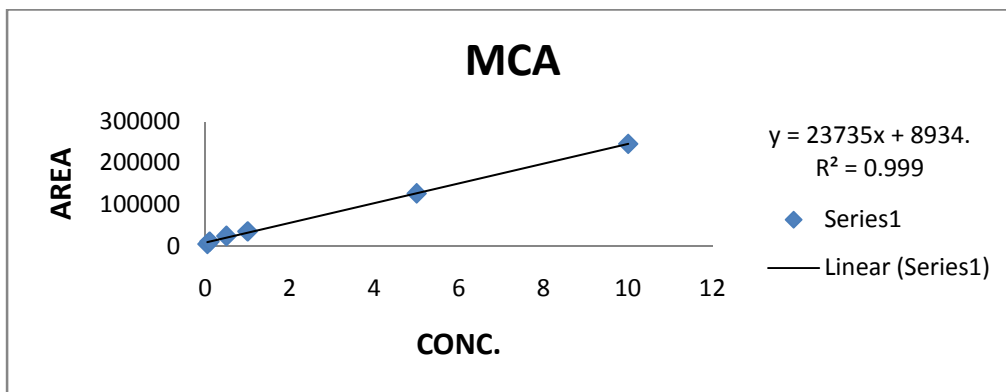


Figure 4: Calibration curve for MCA

Table 2: Accuracy data of CITI and MCA (n=3)

Amount of drug added initially from formulation		Amount of standard drug added (µg/ml)		% recovery ±SD (n = 3)		%RSD	
CITI	MCA	CITI	MCA	CITI	MCA	CITI	MCA
10	1	0	0	100.56±0.92	111.65±1.70	1.00	1.70
10	1	5	0.5	101.40±0.51	109.18±1.52	0.52	1.56
10	1	10	1	103.20±0.49	110.72±0.62	0.50	0.66
10	1	15	1.5	101.71±0.41	111.91±0.51	0.49	0.52

Table 3: Robustness studies of HPLC method (1 µg/ml of CITI & 1 µg/ml of MCA)

Parameter	Method condition	RT		% RSD of peak area	
		CITI	MCA	CITI	MCA
pH	Actual (6.5)	3.81	2.31	0.21	0.42
	6	3.83	2.29	0.87	0.67
	7	3.78	2.32	0.85	0.62
Mobile phase Ratio Acetonitrile: 0.02 M KH ₂ PO ₄	Actual (60:40)	3.81	2.31	0.21	0.42
	55: 45	3.4	2.22	0.72	0.52
	65: 35	3.86	2.32	0.69	0.61
Flow rate	Actual(1ml/min)	3.81	2.31	0.21	0.42
	0.9 ml/min	3.85	2.38	0.30	0.38
	1.1 ml/min	2.9	2.1	0.25	0.41

Table 4: System Suitability Studies

Parameters	CITI	MCA
Asymmetric factor	1.05	1.26
Resolution	2.92	
Theoretical plates	3754.15	4189.52

Table 5: Summary of Validation Parameters of HPLC

Validation parameters	CITI	MCA
Linear range (ng per band)	0.01 - 10	0.05 - 10
Intraday (n = 3)	0.56 – 1.17	0.54 – 1.10
Interday (n = 3)	0.60 – 1.22	0.57 – 1.36
Repeatability of peak area (% RSD, n = 6)	0.84	0.70
Accuracy (%)	99.76 – 101.79%	110.92 – 111.79%
LOD	0.003	0.01
LOQ	0.01	0.05
Robustness	Robust	Robust
Specificity	Specific	Specific

Table 6: Analysis of marketed formulation

Formulation	Labeled Amount (mg)		Amount found (mg)		% of drug found $\pm$ SD (n=3)	
	CITI	MCA	CITI	MCA	CITI	MCA
STORAX PLUS	4.4	3.3	4.45	3.67	101.15 $\pm$ 0.89	111.22 $\pm$ 0.52

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