Simultaneous Estimation of Methylcobalamine and Citicoline in its bulk and Pharmaceutical dosage form by using RP-HPLC method

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Abstract- In the present work a simple, sensitive and specific method, Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) have been developed and validated .To create a cheap, best and easy method than the previous one. ACE C8, column having 250mm x 4.6mm, 5µm in isocratic mode, with mobile phase containing Methanol: Water [40 : 60, v / v] is used. The flow rate is 0.6 ml / min and effluents are monitored at 220 nm. Chromatogram showed peak at a retention time of 3.096 ± 0.008 min for Citicoline and 4.110 ± 0.008 min for Methylcobalamine. The method is validated for system suitability, linearity, precision, accuracy specificity, ruggedness, robustness, LOD and LOQ. Recovery of Citicoline and Methylcobalamine is found to be in the range of 99.22 - 100.11 %. The LOD and LOQ for estimation of Citicoline and Methylcobalamine are found to be 0.2 µg / ml, 0.76 µg / ml, and 0.1664µg / ml, 0.5632 µg / ml respectively. Proposed method can be successfully applied for the quantitative determination of Citicoline and Methylcobalamine in Bulk drug and Pharmaceutical dosage form.

Key words- Citicoline; Methylcobalamine; RP-HPLC; ACE.

INTRODUCTION

SLiterature survey reveals that certain spectrophotometric methods were reported for simultaneous estimation of Citicoline and Methylcobalamine and single method is available for such estimation by RP-HPLC.The utility of the developed method to determine the content of drug in commercial formulation was also demonstrated. Validation of the method was done in accordance with USP and ICH guideline for the assay of active ingredient. The method was validated for parameters like system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection and limit of quantification. This method provides means to quantify the component. This proposed method was suitable for the analysis of Pharmaceutical dosage forms.

ANALYTICAL METHOD DEVELOPMENT

A. Selection of wavelength

A solution of 100 µg/ml of Citicoline and Methylcobalamine were prepared in milliQ water. The resulting solutions were scanned individually on HPLC PDA detector from 190 to 400 nm and also in UV-Visible spectrophotometer. The optimal response for both of them was obtained at 220 nm. Hence the complete method was processed at the wavelength of 220 nm [1].

B. Selection of chromatographic condition

Proper selection of the method depends up on the nature of the sample (ionic/ ionisable/neutral molecule), its molecular weight and solubility. The drugs selected in the present study, were polar in nature. Thus reverse phase HPLC was selected for the initial separation because of its simplicity, suitability, ruggedness and its wider usage [2]

C. Initial separation condition

Preparation of standard solution : 10 mg of Citicoline and 10 mg of Methylcobalamine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give a concentration of 1000 µg/ml. (Stock solution) Further 0.8 ml of Citicoline & 1.28 ml Methylcobalamine were pipetted out from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give a concentration of 80 µg/ml and 128 µg/ml respectively[3]

Preparation of sample solution:

20 Tablets of contents were weighed and triturated in glass mortar. The quantity of powder equivalent to 10 mg of active ingredient present in 20 tablets (1534..5mg) was transferred into a 10 ml clean dry volumetric flask, 7 ml of diluent was added to it and was shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent to give a concentration of 1000 µg/ml and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution).

0.8 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluents up to the mark to give the respective concentrations as par with standard solution.The solution was filtered through 0.45 µm filter before injecting into HPLC system.[5]

Preparation of Placebo:

The amount of powdered inactive ingredient supposed to be present in 20 tablets were accurately weighed and transferred in to 10 ml volumetric flask, 7 ml of diluent was added and shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes and was diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for dilution. 0.8 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 µm filter before injecting into HPLC system.

OPTIMIZED METHOD

Preparation of mobile phase:

A mixture of HPLC Water 400 ml (40 %) and 600 ml of Methanol HPLC (60 %) were mixed and degassed in ultrasonic water bath for 5 minutes and was filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: Mobile phase was used as Diluent.

Chromatographic conditions:

Flow rate **:** 0.6 ml per min

Column **:** C_8 ACE (4.6 x 250mm, 5µm).
Detector wavelength **:** 220 nm
Column oven **:** Ambient
Injection volume **:** 20 µl
Run time **:** 8 min

Test Procedure:
20 µl of the Standard, Sample, Blank and Placebo preparations in duplicate were injected separately into HPLC system and the peak responses for Citicoline and Methylcobalamine were measured. The quantities from the peak area in mg of Citicoline and Methylcobalamine were calculated per tablet taken.

METHOD VALIDATION

1. SYSTEM SUITABILITY
Sample solution of Citicoline and Methylcobalamine were injected three times into HPLC system as per test procedure. The system suitability parameters were evaluated from standard Chromatograms obtained, by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from three replicate injections.

2. LINEARITY

Preparation of stock solution: 10 mg of Citicoline and 10 mg of Methylcobalamine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent to give a concentration of 1000 µg/ml. (Stock solution).

Preparation of Level – I (60ppm of Citicolin & 96ppm of Methylcobalamine):

0.6 ml & 0.96 ml of stock solution was taken in 10 ml of volumetric flask diluted up to the mark with diluent to give the respective concentrations i.e.60 ppm and 96 ppm.

Preparation of Level – II (70 ppm of Citicoline &112 ppm of Methylcobalamine):

0.7 ml & 01.12 ml of stock solution was taken in 10 ml of volumetric flask diluted up to the mark with diluent to give the respective concentrations i.e.70 ppm and

112 ppm.

Preparation of Level – III (80 ppm of Citicoline & 128 ppm of Methylcobalamine):

0.8 ml & 1.28 ml of stock solution was taken in 10 ml of volumetric flask diluted up to the mark with diluent to give the respective concentrations i.e.80 ppm and 128 ppm.

Preparation of Level – IV (90 ppm of Citicoline &144 ppm of Methylcobalamine):

0.9 ml & 1.44 ml of stock solution was taken in 10 ml of volumetric flask diluted up to the mark with diluent to give the respective concentrations i.e.90 ppm and 144 ppm.

Preparation of Level – V (100 ppm of Citicoline &160 ppm of Methylcobalamine)

1.0 ml &1.60 ml of stock solution was taken in 10 ml of volumetric flask diluted up to the mark with diluent to give the respective concentrations i.e.100 ppm and 160 ppm.

Procedure:
Each level solution was injected 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.
The linearity of the method was demonstrated over the concentration range of 60-160µg / ml. Aliquots of five levels were prepared from sample solution and labeled as solution 1, 2, 3, 4 and 5 respectively. The solutions were injected in to HPLC system as per test procedure. The Chromatograms were given A calibration curve was plotted for concentration v/s peak area

3. PRECISION
Preparation of stock solution:
10 mg of Citicoline and 10 mg of Methylcobalamine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution) Further 0.8 ml of Citicoline & 1.28 ml Methylcobalamine was pipette out from the above stock solution into a 10 ml volumetric flask

And diluted up to the mark with diluent to give the concentration of 80 µg/ml and 128 µg/ml respectively.

Intermediate Precision/Ruggedness:
Preparation of stock solution:

10 mg of Citicoline and 10 mg of Methylcobalamine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added ,sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution)
Further 0.8 ml of Citicoline & 1.28 ml Methylcobalamine were pipette out from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 80 µg/ml and 128 µg/ml respectively.

4. ACCURACY

Assay was performed in triplicate for various concentrations of Citicoline and Methylcobalamine equivalent to 50, 100, and 150 % of the standard amount was injected into the HPLC system as per the test procedure.

Preparation of Standard stock solution:

10 mg of Citicoline and 10 mg of Methylcobalamine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution)
Further 0.8ml of Citicoline & 1.28ml Methylcobalamine were pipette out from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 80 µg/ml and 128 µg/ml respectively.

Preparation Sample solutions:
For preparation of 50% solution (With respect to target Assay concentration):

5 mg of Citicoline and 5 mg of Methylcobalamine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added ,sonicated to dissolve it completely and volume was made up to the mark with the same solvent to give the concentration of 500 µg/ml. (Stock Solution).
Further 0.8 ml of Citicoline & 1.28 ml Methylcobalamine were pipette out from the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluents to give the concentration of 40 µg/ml and 64 µg/ml respectively.

For preparation of 100% solution (With respect to target Assay concentration):

10 mg of Citicoline and 10 mg of Methylcobalamine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution)
Further 0.8 ml of Citicoline & 1.28 ml Methylcobalamine were pipette out from the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent to give the concentration of 80 µg/ml and 128 µg/ml respectively.

For preparation of 150% solution (With respect to target Assay concentration): 15.0 mg of Citicoline and 15.0 mg of Methylcobalamine were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give the concentration of 1500 µg/ml. (Stock solution).
Further 0.8 ml of Citicoline & 1.28 ml Methylcobalamine were pipette out from the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent to give the concentration of 120 µg/ml and 192 µg/ml respectively.[6]

5. SPECIFICITY

A) Citicoline and Methylcobalamine identification:
Solutions of Standard and Sample were prepared as per test procedure and injected into the HPLC system.

B) Placebo interference:
A study to establish the interference of placebo was conducted. A sample of placebo was injected into the HPLC system as per the test procedure.

C) Blank interference:
A study to establish the interference of blank was conducted. Diluent was injected into HPLC system as per the test procedure.

6. RUGGEDNESS
The simultaneous estimation of Citicoline and Methylcobalamine was performed by different analysts on different days. The Chromatogram for Day-1, Analyst-1 was presented in and the results for were illustrated. The Chromatogram for Day-2, Analyst-2 was given in and the results were discussed

7. ROBUSTNESS
The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like flow rate and mobile phase composition, temperature variations which may differ but the responses were still within the specified limits of the assay.

8. LIMIT OF DETECTION
For Citicoline
Preparation of 80 µg/ml solution:

10 mg of Citicoline was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added , sonicated to dissolve it completely and volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution).
Further 0.8 ml of the above stock solution was pipetted out into a 10 ml volumetric flask and dilute up to the mark with diluent to give the concentration of 80 µg/ml.

SFor Methylcobalamine:

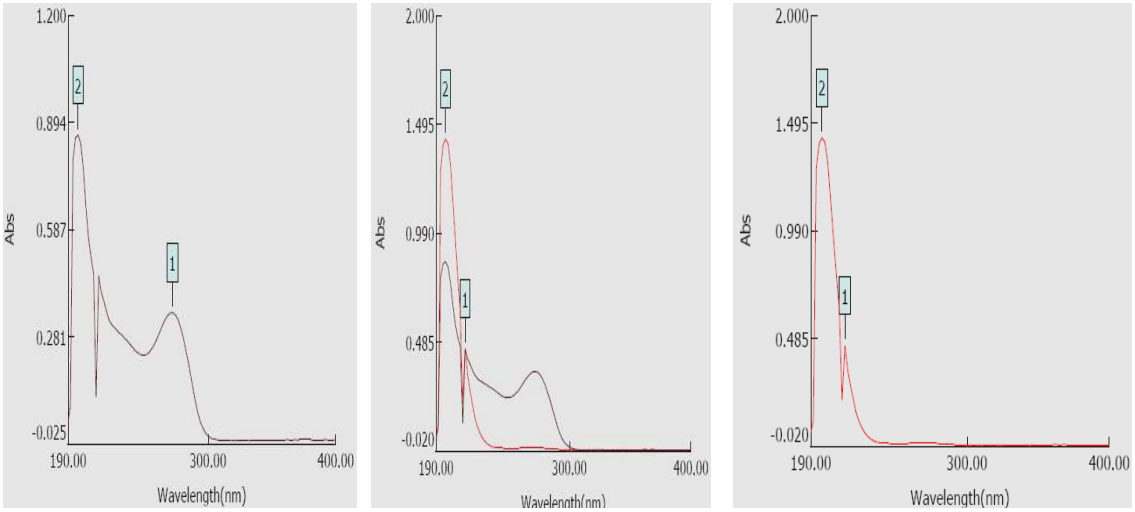
Preparation of 128 µg/ml solution:
10 mg of Methylcobalamine working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution)
Further 1.28 ml of the above stock solution was pipette into a 10 ml volumetric flask and diluted up to the mark with diluent to give the concentration of 128 µg/ml.
The LOD is determined by the formula
LOD = **S**/N
Where
N = Average Baseline Noise obtained from Blank
S = Signal Obtained from LOD solution (0.25% of target assay concentration).[7-8]

9.LIMIT OF QUANTIFICATION
For Citicoline:
Preparation of 80 µg/ml solution:
10 mg of Citicoline was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and make volume up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution).
Further 0.8 ml of the above stock solution was pipette into a 10 ml volumetric flask and diluted up to the mark with diluent to give the concentration of 80 µg/ml.
For Methylcobalamine:
Preparation of 128 µg/ml solution:
10 mg of Methylcobalamine was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and make volume up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution).
Further 1.28 ml of the above stock solution was pipetted into a 10 ml volumetric flask and diluted up to the mark with diluent to give the concentration of 128 µg/ml.[9-10]

LOQ is determined by the following formula:
LOQ =S/N
where
N =Average Baseline Noise obtained from Blank.
S=Signal Obtained from LOQ solution (1% of target assayconcentration)

RESULTS

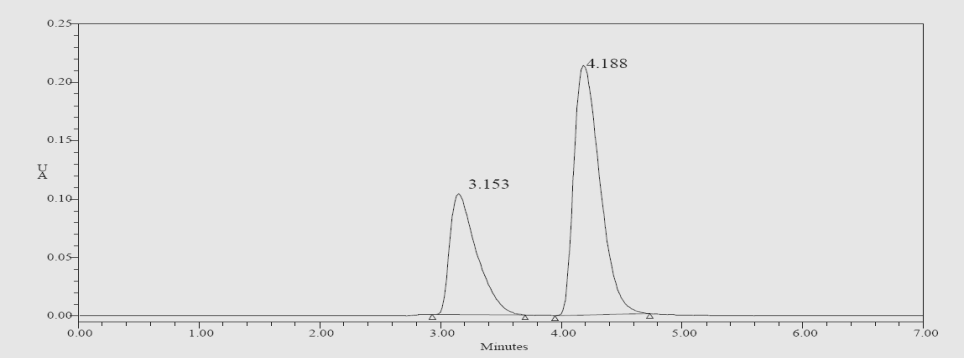
Fig 1: UV Spectrum of Citicoline and Methylcobalamin



METHOD VALIDATION

1. SYSTEM SUITABILITY

Fig 11: Chromatograms for System suitability



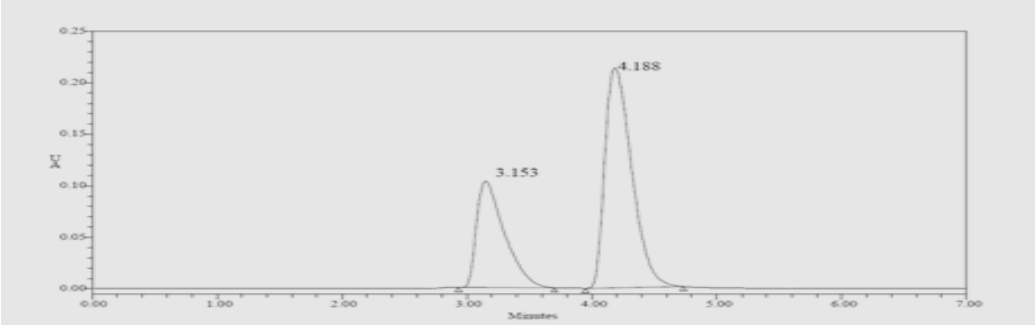


Table: 6 Chromatogram values for System suitability

a) Citicoline **b)Methylcobalamine**

Injection	R_t	Peak Area	USP Plate count	USP Tailing	Injection	R_t	Peak Area	USP Plate count	USP Tailing
1	3.153	1870855	2196.8	1.7	1	4.188	4107813	2843.5	1.5
2	3.152	1851015	2037.2	1.7	2	4.189	4056818	2025.9	1.5
3	3.045	1817754	2014.1	1.7	3	4.134	4010418	2816.2	1.5
Mean	3.116667	1846541			Mean	4.170333	4058350		
SD	0.062067	262831.69			SD	0.03147	88491.65		
% RSD	0.019	1.4			% RSD	0.75	1.9		

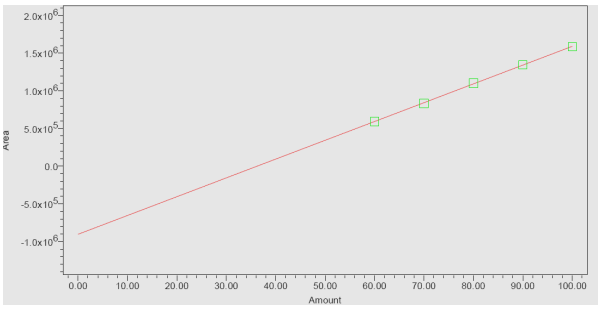
2. LINEARITY

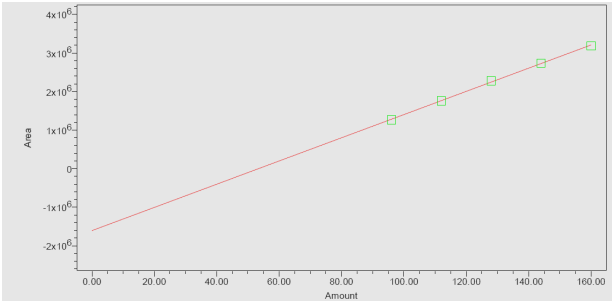
S.No	Linearity Level	Concentration	Area
1	I	60 ppm	593412
2	II	70 ppm	834616
3	III	80 ppm	1101599
4	IV	90 ppm	1348563
5	V	100 ppm	1583765
Correlation Coefficient			0.9997

S.No	Linearity Level	Concentration	Area
1	I	96 ppm	1266663
2	II	112 ppm	1758401
3	III	128 ppm	2280100
4	IV	144 ppm	2736378
5	V	160 ppm	3185591
Correlation Coefficient			0.9995

Linearity results for Citicoline Linearity results for Methylcobalamine

Calibration curve of Citicoline and Methylcobalamin





Calibration parameters for Citicoline and Methylcobalamine

Parameter	**Results for Citicoline**	**Results for Methylcobalamine**
Slope	24946.53	30098.9
Intercept	-903331.4	-1607239
Correlation co-efficient	0.9997	0.9995

3. PRECISION

Sample Chromatogram values for Repeatability

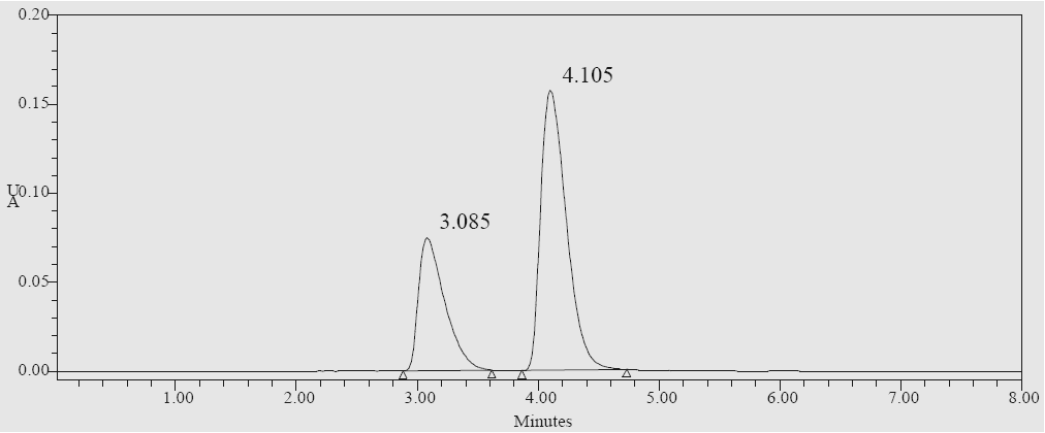
a) Citicoline

Injection No	Peak Area	R_t
1	1870855	3.109
2	1851015	3.104
3	1817754	3.099
4	1823745	3.099
5	1865619	3.099
Avg	1845798	3.102
SD	24087.1	0.0044
% RSD	1.30	0.141

b) Methylcobalamine

Injection No	Peak Area	R_t
1	4107813	4.125
2	4056818	4.122
3	4010418	4.117
4	3991546	4.115
5	4103191	4.115
Avg	4053957	4.1188
SD	52733.9	0.00449
% RSD	1.30	0.109

B)Intermediateprecision(Analyst to Analyst variability):



Chromatograms for Intermediate precision

Sample Chromatogram values for intermediate Precision

a) Citicoline

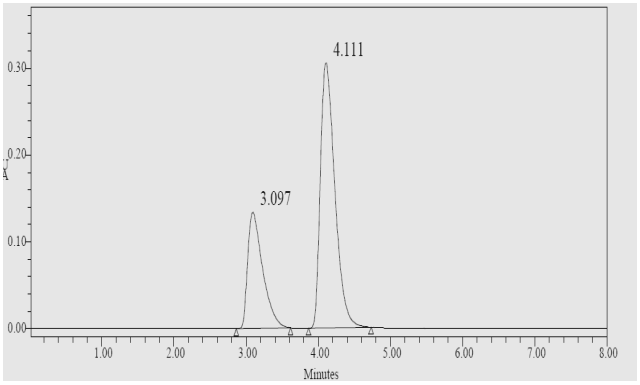
Injection No	Peak Area	R_t
1	1121577	3.085
2	1129193	3.082
3	1134378	3.094
4	1132712	3.078
5	1125645	3.074
Mean	1128701	3.082
SD	5211.9	0.0076
% RSD	0.46	0.24

b) Methylcobalamine

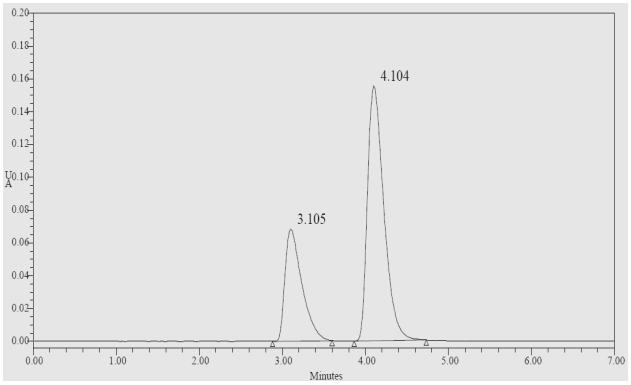
Injection No	Peak Area	R_t
1	2319029	4.105
2	2326003	4.101
3	2332424	4.114
4	2345223	4.094
5	2336364	4.088
Mean	233009	4.100
SD	10419.6	0.0100
% RSD	0.45	0.24

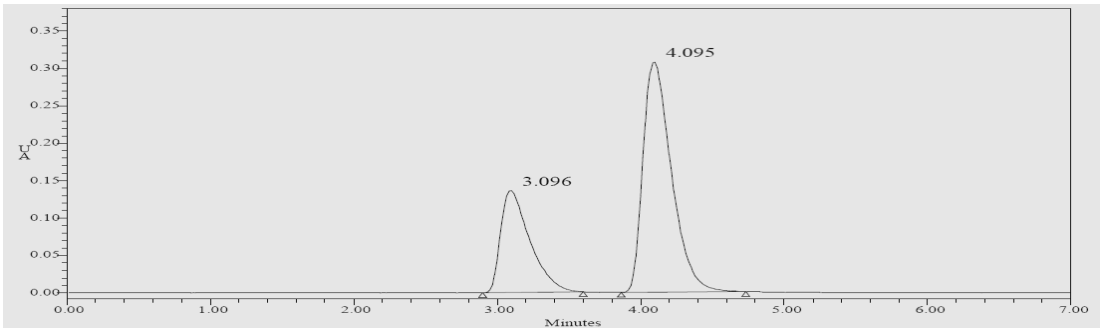
4. ACCURACY

Standard Chromatogram for Accuracy



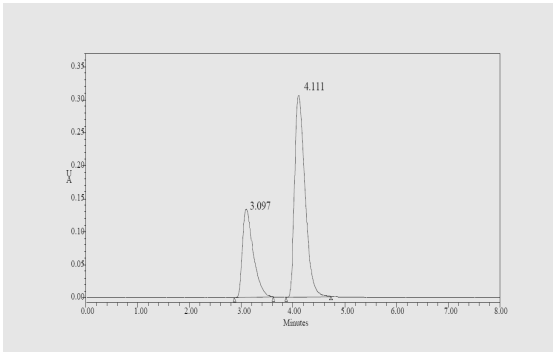
Chromatograms for Acc 50% & Acc 100%

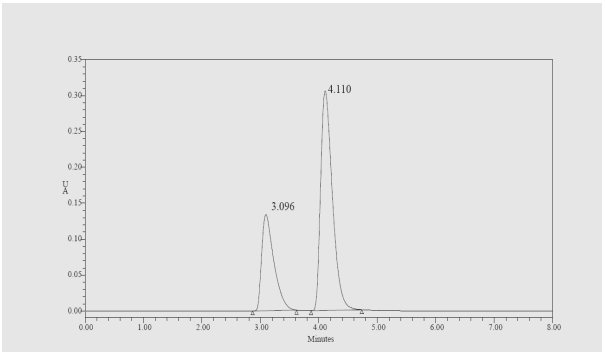




5. SPECIFICITY

Standard and sample Chromatogram for Citicoline and Methylcobalamine Identification





7. ROBUSTNESS

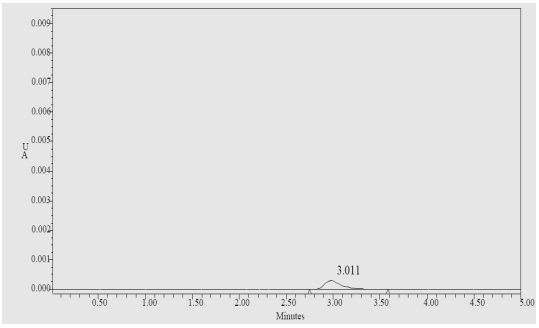
Robustness results for Citicolin (flow rate)& Methylcobalamine (flow rate):

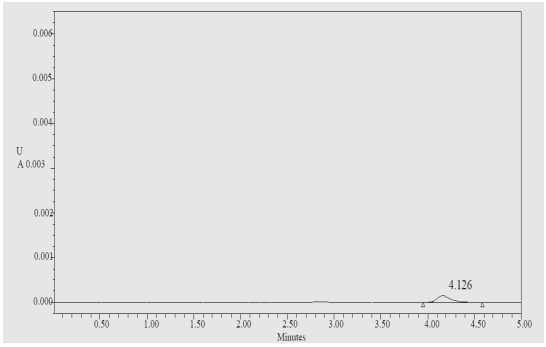
S.No	Drug	Flow Rate ml/min		
		0.5 ml/min	0.6 ml/min	0.7 m l/min
1	Citicoline	3.915	3.109	2.519
USP Plate count		2196.8	2037.2	2014.1
USP Tailing		1.7	1.7	1.7

S.No	Drug	Flfow Rate ml/min		
		0.5 ml/min R_t	0.6 ml/min R_t	0.7 m l/min R_t
1	Methylcobalamine	5.222	4.125	3.337
USP Plate count		2843.5	2025.9	2816.2
USP Tailing		1.5	1.5	1.5

8. LIMIT OF DETECTION (LOD)

Chromatogram for Citicoline:





Chromatogram For Methylcobalamine:

CONCLUSION

For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation step. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool. Citicoline was freely soluble in water and insoluble in alcohol and acetonitrile. Methylcobalamine was freely soluble in water and sparingly soluble in alcohol. Methanol and Water was chosen as the mobile phase. The run time of the HPLC procedure was 8 minutes. The method was validated for system suitability, linearity, precision, accuracy, specificity, ruggedness robustness, LOD and LOQ. The system suitability parameters were within limit, hence it was concluded that the system was suitable to perform the assay. The method shows linearity between the concentration range of 60-160 µg / ml. The % recovery of Citicoline and Methylcobalamine were found to be in the range of 99.22 % 100.11 %. As there was no interference due to excipients and mobile phase, the method was found to be specific.

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