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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF CITICOLINE IN BULK AND PHARMACEUTICAL DOSAGE FORM BY RP-HPLC METHOD

Sunil Mekala*[1], S. K. Mohammed Samiulla[1], S. Arun Teja[1], S. K. Sahera Bhanu[1], M. Bhavani[1], P. Bhagya Lakshmi[1]

[1*]Department of Pharmaceutics, Vishwa Bharathi College of Pharmaceutical Sciences, Perecherla, Guntur, Andhra Pradesh, India.

ABSTRACT

An analytical method has been developed and validated for estimation of citicoline sodium in pharmaceutical dosage form (tablet) by RP-HPLC. The chromatography was carried out isocratically by a BDS Hypersil C18 column (250 x 4.6mm, 5µm) with a mixture of buffer (Potassium di hydrogen phosphate and Tetra butyl ammonium hydroxide): Methanol in the ratio of 95:05 v/v as mobile phase.

KEYWORDS

Citicoline sodium, Chromatography, Stationary phase, Mobile phase and Reversed ph.

Author for Correspondence:

Sunil Mekala,

Department of Pharmaceutics,

Vishwa Bharathi College of Pharmaceutical Sciences,

Perecherla, Guntur, Andhra Pradesh, India.

Email: dr.sunilmekala@gmail.com

INTRODUCTON

CT (CDP-choline) stimulates the biosynthesis of cerebral phosphatidylcholine, main structure component of the phospholipids of the neuronal membrane. CT increase the neurotransmission levels because it favours the synthesis and production speed of dopamine in the striatum, acting then as dopaminergic agonist through the inhibition of tyrosinhydroxilase. CT behaves like a presypnatic cholinergic agent in favouring the synthesis of acetylcholine. Citicoline improves the neuronal metabolism in those cases where there is a neuronal deterioration due to degenerative, toxic or ischemic cause[1]. In the present study, simple, sensitive, efficient, reliable and accurate method of analysis to determine citicoline sodium in tablet

dosage form (Cognipil-500) was developed and validated using RP-HPLC.

MATERIAL AND METHODS

Method development and validation of citicoline in pharmaceutical dosage forms. Isocratic Reversed phase HPLC method for the estimation of Citicoline in pharmaceutical dosage forms. In the present investigation, new Isocratic RP-HPLC method has been developed for the estimation of Citicoline in pharmaceutical dosage forms, which is simple, sensitive, accurate, precise and rapid.

Instrumentation

Quantitative HPLC was performed on Shimadzu prominence Isocratic HPLC system with LC20 AT pump, SPD-20A detector, Sphinchrom CFR software, XDB C_8(4.6 x150mm, 5µm). Chromatographic separations were achieved using a guard column and analytical column C_8(4.6x 150mm, 5µm). The mobile phase consisting of phosphate buffer of pH 3.0 and acetonitrile in the ratio of 60:40 v/v was passed through 0.45µ membrane filter and degassed by ultrasonication under vacuum before use. The flow rate was maintained at 0.5ml/min and the effluent was monitored at 272nm. The injection volume was 20µl. All separation was performed at an ambient temperature.

Reagents and chemicals used

a) HPLC grade Acetonitrile b) HPLC grade water c) Potassium dihydrogen phosphate d) Citicoline working standard

Preparation of P^H 3 buffer solution

About 6.8 gm of potassium dihydrogen phosphate was transferred into a 1000mL volumetric flask containing 700mL of water. The contents were sonicated for about 5 minutes and the volume made upto 1000mL with water. This solution was mixed and pH was adjusted to 3.0 with orthophosphoric acid and filtered through 0.45µ nylon filter.

Preparation of mobile phase

Accurately measure a 600ml of Phosphate buffer of pH 3.0 (60%) and 400ml of Acetonitrile HPLC Grade (40%) to get the final concentration in the ratio of 60:40 v/v and degas in ultrasonic water bath for 5 minutes. Filter through 0.45µ membrane filter under vacuum filtration.

Preparation of Standard Solution

Weigh accurately about 25mg of Citicoline (working standard solution) and transferred into a 100ml volumetric flask, add about 70ml of diluents (phosphate buffer and acetonitrile in ratio of 60:40) and sonicate to dissolve it completely and make the volume up to the mark with the same solvent. (Stock Solution 250µg/ml). From this stock solution 2ml is taken into 10ml volumetric flask and make the volume up to the mark with diluents to get the final concentration of 50µg/ml. A typical chromatogram was shown in Figure No.1. Prepare different concentrations of standard solutions containing 10, 25, 50, 75, 100, 125µg/ml and injected into the column at a flow rate 0.5ml/min. The peak areas of the different drug concentrations were calculated.

Preparation of Sample solution

10 Tablets were weighed and powdered. An accurately weighed portion of this powder equivalent to 25mg of Citicoline was transferred in to 100ml volumetric flask. Add about 70ml of diluents and sonicate to dissolve it completely for 20 min and make upto the mark with the diluents (phosphate buffer and acetonitrile in ratio of (60:40v/v). Mix well and filter through 0.45µm membrane filter paper. Further pipette out 1ml of the above solution into a 10 ml volumetric flask and dilute upto the mark with diluents. A typical chromatogram was shown in Figure No.2. The sample solution of 20µl is injected five times into the column at a flow rate of 0.5 ml/min, the peak area of the drug were calculated.

METHOD DEVELOPMENT

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate wave length and choice of stationary and mobile phases. The following studies were conducted for this purpose.

Detection of analytical wavelength

From the standard stock solution further dilutions were done using mobile phase and scanned over

the range of 200-400nm and the spectra was overlain it was observed that 272nm is λ_{max} for Citicoline and the wavelength suitable for Citicoline was preferred.

Selection of stationary phase

Preliminary development trials have been performed with octadecyl columns of different types, configurations and from different manufacturers.

Selection of mobile phase

The solution of Citicoline was injected into the HPLC system and run in different solvent systems. Different mobile phases containing methanol, water and acetonitrile in different proportions were tried and finally were selected as a Phosphate buffer of pH 3 and acetonitrile (60:40 v/v) appropriate mobile phase which gave good resolution and acceptable peak parameters for Citicoline.

Flow rate

Flow rates of the mobile phase were changed from 0.5-1.0mL/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents.

VALIDATION OF THE PROPOSED METHODS

Validation of developed analytical method was performed as per ICH guidelines over the specificity, linearity, precision, accuracy, robustness, system suitability, limit of detection, limit of quantification and solution stability.

Specificity

Specificity is the ability to assess unequivocally the analytic in the presence of components that may be expected to be present. The specificity of method was determined by comparing the chromatograms of blank, standard and sample. The specificity results are shown in Table No.2.

Linearity

Linearity was evaluated by analysis of standard solutions of Citicoline. Six standard concentrations of citicoline ranging from 50-150% of target concentration (10 to125µg/ml) were prepared and assayed in triplicate. The response was read at 282nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and a linearity plot of concentration over the mean peak area was constructed. The regression of the plot was computed by least square regression method. Linearity results are presented in Table No.3 and linearity plot was shown in Figure No.9.

Precision

The precision of the method was determined by intraday and inter day on three different days. Precision was assessed by performing replicate analyses of quality control samples against calibration standard. The results obtained for inter and intraday variations were shown in the Table No.4 and Table No.5.

Accuracy

The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed injection solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at 50%, 100% and 150% level. The solutions were analyzed in triplicate at each level as per the proposed method. The percent recovery and % RSD at each level was calculated and results are presented in Table No.6.

Robustness

The robustness of the proposed method was estimated by changing flow rate of the mobile phase, pH of the buffer. Samples of Citicoline at 150µg/mL concentration were analyzed under these changed experimental conditions. Results are presented in Table No.7 and Table No.8.

System suitability

To ensure the validity of the analytical procedure, A System suitability test was established. Data from six injections of 10µl of working standard solution of citicoline were used for the evaluation of system suitability parameters like number of theoretical plates, retention time, area, peak ratio by LC solution software. The system suitability parameters are given in Table No.11.

Limit of detection and Limit of quantification

Limit of detection (LOD) is defined as the lowest concentration of analyte that gives a detectable response. Limit of quantification (LOQ) is defined

as the lowest concentration that can be quantified reliably with a specified level of accuracy and precision. For this study, six replicates of the analyte at lowest concentration were measured and quantified. The LOD and LOQ of Citicoline are given in Table No.9.

Assay

10 tablets each containing 5mg were weighed and powdered, an accurately weighed portion of the powder equivalent to 5mg of Citicoline was transferred to 100ml volumetric flask containing 50ml of mobile phase. The contents of the flasks were sonicated for 20 min to dissolve Citicoline and made upto the volume with mobile phase and resulting mixture filtered through 0.45µm membrane filter paper. 2.5ml of the above solution was diluted to a 100ml with mobile phase. This solution (20µl) was injected 5times in to the column. The mean values of peak areas of five such determinations were calculated and the content in the tablets was quantified using regression equation obtained above. The same procedure was followed for the estimation of Citicoline in other commercially available tablet dosage forms. The results are given in the Table No.10.

RESULTS AND DISCUSSION

Optimized chromatographic conditions

Chromatographic conditions as optimized are shown in Table No.1. These optimized conditions were followed for the determination of Citicoline in bulk samples and tablet formulation.

METHOD DEVELOPMENT DATA

Specificity

It was found that there is no interference due to excipients in the tablet formulation and also found good correlation between the retention times of standard and sample.

LOD and LOQ

The LOD and LOQ of the proposed method at 272nm were found to be 0.000287µg/ml, 0.000457µg/ml respectively, which indicated the method can be used for detection and quantification of Citicoline over a wide range of concentrations

Discussion

Citicoline can be analyzed by using proposed HPLC method both in pure drug and its pharmaceutical dosage forms. The aim of the study was to develop a chromatographic procedure for the analysis of Citicoline in pure drug and pharmaceutical dosage forms. The column used in this method XDB C8 (4.6 x150mm, 5µm) showed a high resolution separation. Moreover the mobile phase was modified. The influence of different organic solvents in the mobile phase was studied aiming to establish the best experimental conditions for the analysis of Citicoline.

The Detection of the Citicoline was set at 282nm. The retention time for Citicoline was 2.005min for a run time of 4min. Each of the samples was injected 5 times. A total 20µl volume of each sample was injected in to the column. The peak areas were shown in Table No.6. Linearity was obtained in the concentration range of 10-125µg/ml. The results showed that the methods have reasonable precision. The standard calibration curve of Citicoline was constructed by plotting the peak area versus respective drug concentration. The correlation coefficient was 0.999 indicating excellent linearity. The system was validated for system suitability, precision, accuracy, linearity, ruggedness, and robustness.

The optical characteristic such as Beer's law limit was calculated and correlation coefficient(r) obtained from different concentrations and the results were summarized in Table No.7. The percent relative standard deviation and percent range of errors (0.05 and 0.01 level of confidence limits) was calculated and summarized in Table No.7.

The precision of the method was determined by intraday and inter day on three different days. Precision was assessed by performing replicate analyses of quality control samples against calibration standard. The results obtained for inter and intraday variations were shown in the Table No.4 and Table No.5.

The Accuracy of the method was determined by the Recovery studies, carried out at three different levels 50%, 100%, 150%. The results were shown

in Table No.6. About 98% -102% Citicoline could be recovered from the pre-analysed sample indicating the high accuracy of the proposed HPLC method. No interfering peaks were found in chromatograms due to tablets excipients.

The robustness study was carried out by bringing intentional changes in flow rate (0.7-0.9ml) and change of mobile phase composition (<10 and >10). The difference in the results obtained by this study insignificant variations were observed in the Table No.7 Table No.8.

The ruggedness of the method was demonstrated by analysis of the sample by two different analysts using two different sets of instruments (AGILENT, C-8 and ACE, C-8). The RSD of two sets of data (0.03 and 0.2) indicates the ruggedness of the method. The proposed RP-HPLC method was found to be simple, precise, highly accurate, specific and less time consuming which can be used for routine quality control tests of Citicoline.

SUMMARY

The following is the summary of method validation study conducted for Method development of citicoline b simultaneous estimation by RP-HPLC Method.

Quantitative HPLC was performed on Shimadzu prominence Isocratic HPLC system with LC20 AT pump, SPD-20A detector, Sphinchrom CFR software, XDB C_8 (4.6 x150mm, 5µm).

The mobile phase consisting of phosphate buffer of pH 3.0 and acetonitrile in the ratio of 60:40 v/v.

The column used in this method XDB C8 (4.6 x150mm, 5µm) showed a high resolution separation. Moreover the mobile phase was modified.

The Detection of the Citicoline was set at 282nm. The retention time for Citicoline was 2.005min for a run time of 4min.

Linearity was obtained in the concentration range of 10-125µg/ml.

The standard calibration curve of Citicoline was constructed by plotting the peak area versus respective drug concentration. The correlation coefficient was 0.999 indicating excellent linearity.

The system was validated for system suitability, precision, accuracy, linearity, ruggedness, and robustness.

About 98%-102% Citicoline could be recovered from the pre-analysed sample indicating the high accuracy of the proposed HPLC method. No interfering peaks were found in chromatograms due to tablets excipients.

The ruggedness of the method was demonstrated by analysis of the sample by two different analysts using two different sets of instruments (AGILENT, C-8 and ACE, C-8). The RSD of two sets of data (0.03 and 0.2) indicates the ruggedness of the method.

Table No.1: Optimized chromatographic conditions for estimation of Citicoline in pharmaceutical dosage forms

Mobile phase	Buffer: Acetonitrile = 60:40 v/v
Pump mode	Isocratic
pH of Buffer	2.5 ± 0.05
Column	XDB C_8(4.6 x150mm,5µm)
Column Temp	Ambient
Wavelength	272nm
Injection Volume	20µl
Flow rate	0.5mL/min
Typical t_R	2.39 min
Run time	4 min

Table No.2

S.No	Name of solution	Retention time (min)
1	Blank	No peaks
2	Standard	2.395
3	Sample	2.395

Linearity

Table No.3: Linearity

S.No	Level	Concentration of Citicoline (µg/mL)	Mean peak area
1	Level -1	10	385691
2	Level -2	25	1017593
3	Level -3	50	1978654
4	Level -4	75	2930327
5	Level -5	100	3928842
6	Level -6	125	4706494
7	Slope		38126
8	Intercept		38417
9	Correlation Coefficient		0.9999
10	Range: 50 to 150% of target concentration (i.e. 10to 125µg/mL)		

Table No.4: Intraday precision for Citicoline assay in pharmaceutical dosage forms by the proposed HPLC method

S.No	Concentration (µg/ml)	Peak area (Intraday)	Mean (n=5)	SD	CV
1	25	1067308	1039632	19267.4	0.0185
2	25	1052512			
3	25	1027692			
4	25	1024599			
5	25	1026049			

Table No.5: Inter day precision for Citicoline assay in pharmaceutical dosage forms by the proposed HPLC method

S.No	Concentration (µg/ml)	Peak area (Intraday)	Mean (n=5)	SD	CV
1	25	1292741	1300761.1	4737.1	0.0036
2	25	1301638			
3	25	1301614			
4	25	1305345			
5	25	1302471			

Table No.6: Accuracy and recovery of citicoline using proposed HPLC method

S.No	% Concentration (at Specification Level)	Peak Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
1	50%	385581	10.2	9.0	96.9%	98.5%
2	100%	1022684	24.2	23.9	98.8%	
3	150%	1597374	37.4	37.34	99.8%	

Table No.7: Robustness study with regards to Flow rate variations

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.5	1936	1.4
2	0.6	1636	1.4
3	0.7	1422	1.2

Table No.8: Robustness study with regards to to change in Mobile phase composition

S.No	Change in organic composition in the Mobile phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	1145	1.1
2	Actual	1963	1.4
3	10% more	1688	1.5

Table No.9: LOD and LOQ

S.No	Parameter	Measured value (µg/mL)
1	Limit of detection	0.000287
2	Limit of quantification	0.000457

Table No.10: Mean (±SD) amount of Citicoline hydrochloride in tablet dosage forms by the proposed HPLC method

Brand of tablet	Labeled amount of drug (mg)	Mean (±SD) amount found (mg) by the proposed method (n=5)	Mean (±SD) % labeled amount (n=5)
T_1	500	498	99.6
T_2	500	492	98.4

Table No.11: Optical characteristics of Citicoline parameters Method

Retention time (t) (min)	2.3
% RSD	0.036
No. of Theoretical plates (n)	1963
Tailing factor	1.4
LOD (µg/ml^{-1})	0.000287
LOQ(µg/ml^{-1})	0.000457
Plates per meter (N)	25.3933
HETP (mtr)	0.0764
Regression equation (Y*) Slope (b)	37874.168
Intercept (a)	61007.75
Correlation coefficient (r)	0.999
Standard Error of estimation	0.1080
Range of errors Confidence limits with 0.05 level	±0.4372
Confidence limits with 0.01 level	

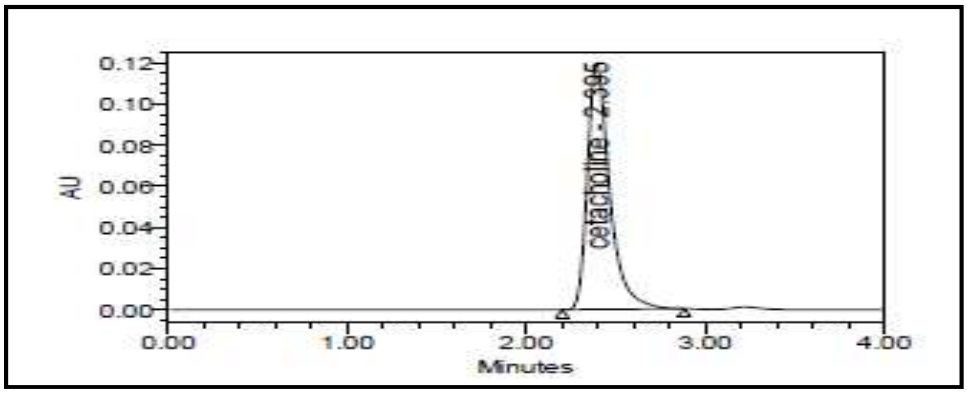

Figure No.1: Typical chromatogram of Standard Citicoline

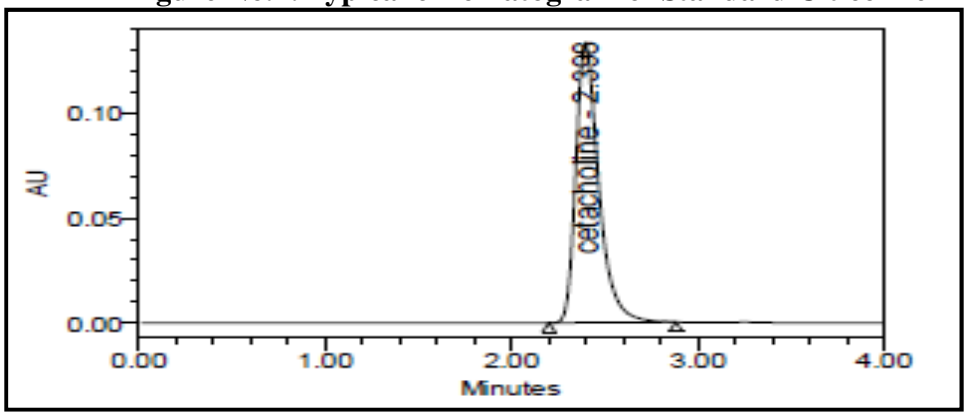

Figure No.2: Typical chromatogram of Citicoline formulation

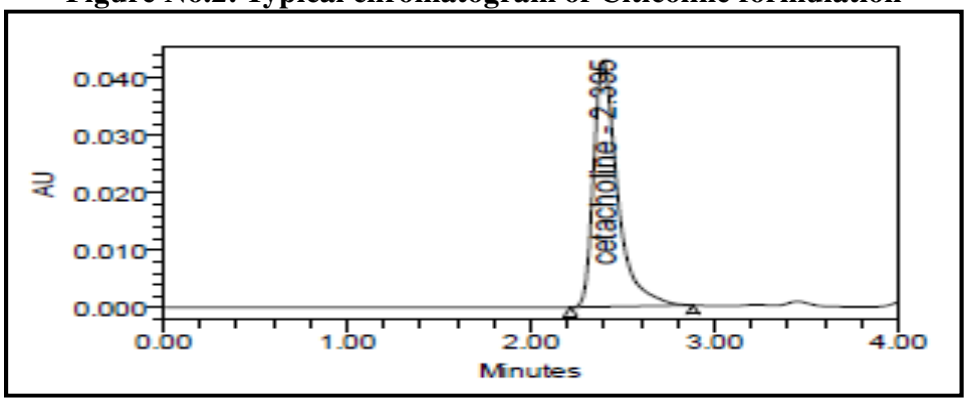

Figure No.3: Typical chromatogram of 10µg/ml of Citicoline

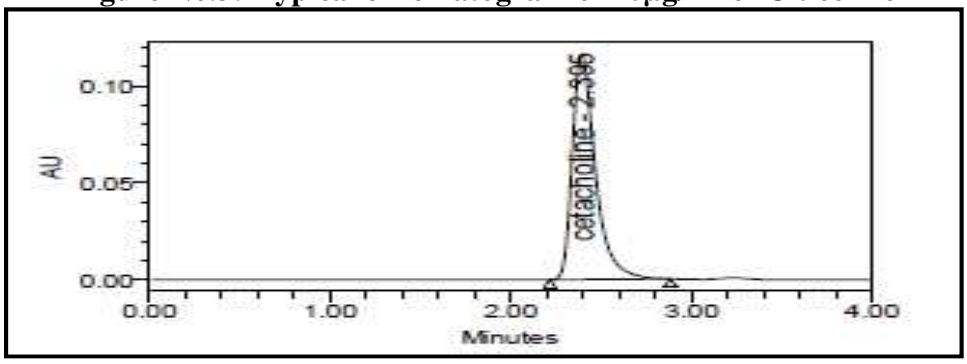

Figure No.4: Typical chromatogram of 25µg/ml of Citicoline

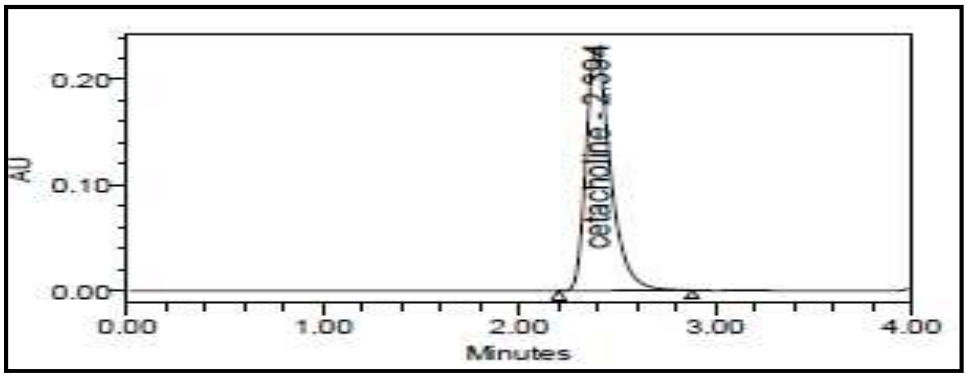

Figure No.5: Typical chromatogram of 50µg/ml of Citicoline

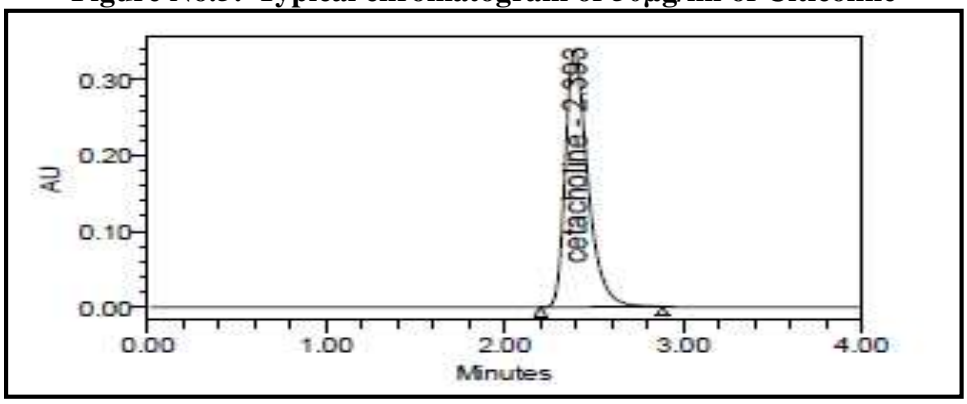

Figure No.6: Typical chromatogram of 75µg/ml of Citicoline

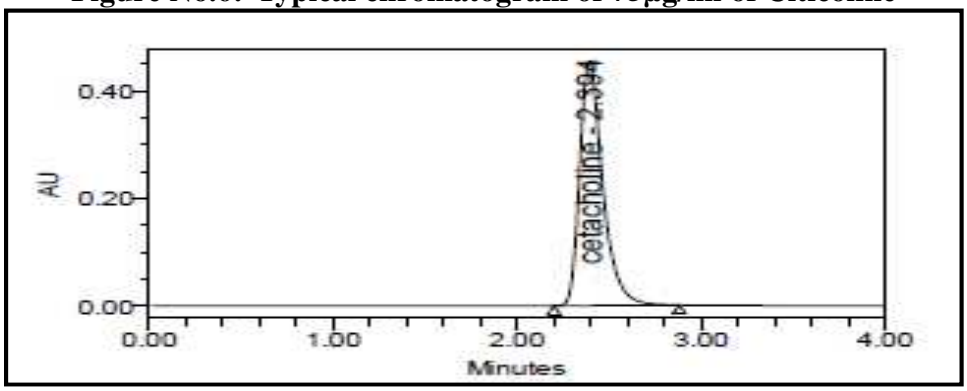

Figure No.7: Typical chromatogram of 100µg/ml of Citicoline

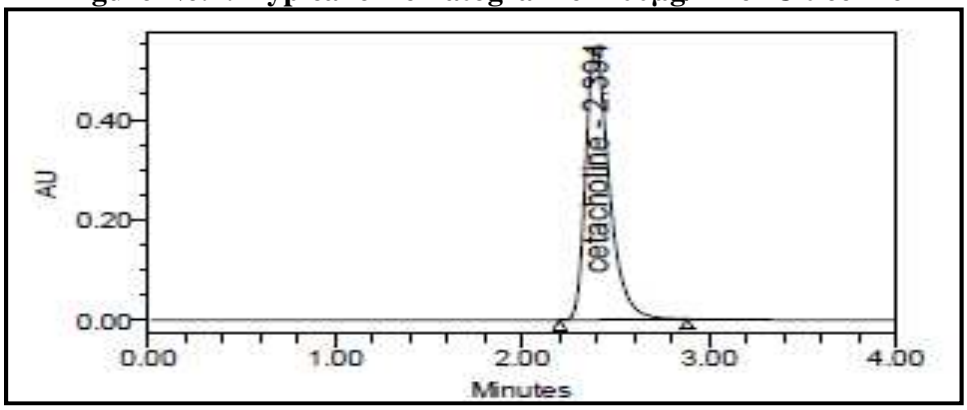

Figure No.8: Typical chromatogram of 125µg/ml of Citicoline

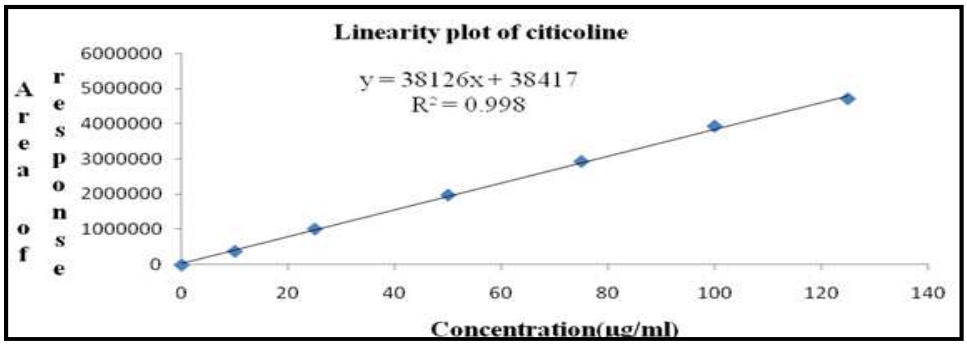

Figure No.9: Calibration curve of Citicoline (RP-HPLC)

CONCLUSION

The proposed RP-HPLC method determination for estimation of citicoline in pure and pharmaceutical dosage forms is found to be specific, linear and accurate in the specified range. The method is found to be precise and robust. System suitability test is established and recorded. Hence, this method stands validated and can be used for routine analysis at laboratory.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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