Research Article

Development and Validation of Analytical Method for Simultaneous Estimation of Citicoline Sodium and Preservative Methyl Paraben in Liquid Oral Formulation by RP-HPLC

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ABSTRACT

A rapid, accurate, specific, linear and sensitive reverse-phase HPLC method has been developed and validated for simultaneous determination of citicoline (CITI) and preservative methyl paraben (MetP) in oral drop formulation. Chromatographic separation was carried out on (Merck) C_8 column (250mm x 4.6mm, 5µm particle size) using a mobile phase consisiting of 0.1 M monobasic potassium phosphate: Methanol (70: 30, v/v). Flow rate was maintained at 1.5 ml/min and 30ºC column temperature with the detection wavelength at 294 nm. The calibration curve was found to be linear over the range of 80-120 ppm for citicoline sodium and 8-12 ppm for methyl paraben with a square correlation coefficient of 0.999 and 0.999 for CITI and MetP respectively. Citicoline sodium and methyl paraben were resolved on the stationary phase and the retention times were found to be 2.06 and 14.68 minutes for citicoline sodium and methyl paraben respectively. The percentage purity of Citicoline sodium and methyl paraben was found to be >99.0%. The method was found to be simple, accurate, precise, and reproducible which were within the acceptance limit according to ICH guidelines and easy to apply, making it very suitable for routine analysis of citicoline sodium and methyl paraben in oral drop formulation.

Keywords: Citicoline Sodium, methyl paraben, RP-HPLC, validation

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INTRODUCTION

Citicoline (CDP- choline) stimulates the biosynthesis of cerebral phosphatidylcholine, main structure component of the phospholipids of the neuronal membrane. Citicoline increase the neurotransmission levels because it favours the synthesis and production speed of dopamine in the striatum, acting then as dopamine in the striatum, acting then as dopaminergic agonist through the inhibition of tyrosinhydroxilase. Citicoline 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]. Citicoline Sodium is white crystalline, somewhat hygroscopic powder. Chemically it is Cytidine 5'(trihydrogendiphosphate) p[2 (trimethylammonio) ethyl] ester inner salt [2]. It is freely soluble in water but insoluble in ethanol, acetone and chloroform [3]. Citicoline is derivative of choline and cytidine involved in biosynthesis of lecithin. It is claimed to increase blood flow and oxygen consumption in the brain and has been given by injection in the treatment of cerebrovascular disorders. It is primarily used in pharmacotherapy of brain insufficiency and other related neurological disorder viz., as stroke, brain trauma and parkinsonism's disease [4].

Methyl Paraben is an effective preservative in many types of pharmaceutical formulations. Chemically it is Methyl 4-hydroxybenzoate. It is white crystalline powder, easily soluble in diethyl ether, acetone and slightly soluble in cold water, hot water [5].

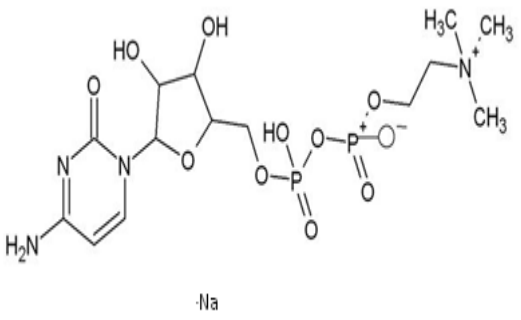


Figure 1: Chemical Structure of Citicoline Sodium

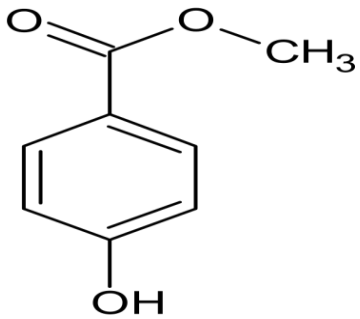


Figure 2: Chemical structure of methyl paraben

UV-visible spectrophotometric method using standard absorptivity value for determination of citicoline sodium and methyl paraben individually or with other combinations in pharmaceutical dosage form were also reported [6-9]. A chromatography methods for determination of citicoline sodium and methyl paraben individually or with other combinations in pharmaceutical dosage form were also reported [10-14]. Since none of RP-HPLC method reported for the simultaneous estimation of citicoline sodium and methyl paraben in liquid oral drop formulation. An aim of the present work was to develop a simple, accurate, precise, reproducible and comparatively economical RP- HPLC method for simultaneous estimation of citicoline sodium and methyl paraben in liquid oral drop formulation.

MATERIALS AND METHODS

Instrumentation

Quantitative HPLC was performed on a high pressure isocratic High Performance Liquid Chromatography (Analytical Technology Limited., SPD-20AD, HPLC workstation) with reciprocating pump, programmable variable wavelength UV detector and Merck C_8 column (250mm x 4.6 mm and particle size 5 µm).

Standards and Chemicals

Citicoline Sodium and Methyl Paraben were kindly provided by Unijules Life Science Ltd. (Nagpur, Maharashtra, India). All the chemicals used were of HPLC grade. Commercial Citicoline Sodium oral drop was also provided by Unijules Life Science Ltd. (Nagpur, Maharashtra, India).

Chromatographic conditions

The chromatographic condition for method was optimized is shown in Table 1.

The composition of the mobile phase was 0.1 M monobasic potassium phosphate: Methanol (70: 30, v/v) was selected after several permutation and combination as shown in Table 2. The mobile phase was filtered through membrane filter 0.45 µm and vacuum degassed. The mobile phase flow rate was set at 1.5ml/min. All standard and assay samples were filtered through membrane filter before injection. After equilibration of column with the mobile phase indicated by a stable baseline, aliquots of sample (20 µl) were injected and the total run time was kept 20 min. The absorbance of the eluent was monitored at 294 nm with a detection sensitivity of 0.1000 aufs. Working standard solution and test solution were injected and chromatograms have been reproduced in following (**Fig. 3 and Fig. 4**).

Table 1: Optimized chromatographic conditions

PARAMETERS	OPTIMIZED CONDITION
Mobile phase	0.1 M monobasic potassium phosphate: Methanol (70: 30, v/v)
Flow rate	1.5ml/min
Run time	20 min
Column	Merck C_8 (250 mm x 4.6 mm, 5µm particle size)
Temperature	Ambient
Wavelength	294 nm
Injection volume	20 µl

Preparation of Analytical Solutions

Preparation of 0.1M Monobasic Potassium Phosphate

Prepared by dissolving 13.6 g of potassium dihydrogen phosphate in 1000ml of distilled water.

Preparation of mobile phase

The mobile phase was prepared by mixing 700 ml 0.1M monobasic potassium dihydrogen phosphate and 300 ml of Methanol and degassed in ultrasonic water bath for 2 minutes. Filter through 0.45µ filter under vacuum filtration.

Preparation of standard stock solution

Standard stock solutions of CITI and MetP was prepared by dissolving 10 mg of drug in 10 ml from the resulting stock solution with mobile phase to get concentration of 1000 µg/ml. Working standard solutions were freshly prepared daily by appropriate dilution of the stock solutions with mobile phase.

Preparation of sample solution

Take 1ml of oral drop equivalent to 100 mg of CITI and 10 mg of MetP [1ml of oral drop contain 100 mg CITI and 2 mg MetP, ratio is about (50:1), by adding pure MetP of 8 mg in 1ml of oral drop to make the final ratio (10:1)] was transferred in 100ml volumetric flask and dissolved in the mobile phase by shaking vigorously for 10 minutes and volume was made to the mark with mobile phase. The solution was filtered through 0.45µ membrane filter. Further the dilutions were made with the mobile phase to get the final concentration of 10 µg/ml of CITI and 1 µg/ml of MetP.

Procedure: Equal volumes (20 µL) of standard and sample solutions were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e., peak area of major peaks were measured. The content of CITI and MetP was calculated by comparing a sample peak area with that of standard.

Calculation and formula:

$$\% \text{ Estimation} = \frac{At}{As} \times \frac{Ds}{Dt} \times \frac{Ws}{Wt} \times 100$$

Where,

At = Area count for sample solution
As = Area count for standard solution
Ds = Dilution factor for sample
Dt = Dilution factor for standard
Ws = Weight of standard (mg)
Wt = Weight of sample (mg)

The results are shown in (**Table 2**).

Table 2: Data of analysis of oral drop formulation by proposed method

S. N.	Amount of oral drop taken for assay (ml)	Amount of drugs estimated (mg/ml)		% of Labelled Claim	
		CITI	MetP	CITI	MetP
1	1.0	101.05	10.07	101.05	100.5
2	1.0	101.03	10.08	101.03	100.8
3	1.0	101.19	10.07	101.19	100.7
4	1.0	101.94	10.07	101.94	100.7
5	1.0	101.89	10.07	101.89	100.7

Statistics

Drug	Mean (%)	± S.D.	R.S.D. (%)
CITI	101.42	0.4564	0.4500
MetP	100.68	0.1095	0.1088

Running of mobile phase

The mobile phase was prepared and injected into RP-HPLC system and the chromatogram of baseline was obtained after system stabilization.

Optimization of mobile phase

A very sharp peak was given by citicoline sodium and methyl paraben with an appropriate and low retention time with mobile phase ratio of (0.1 M monobasic potassium phosphate: Methanol (70: 30,

v/v) which can be used for routine analysis, so it was an optimized condition of the analytical method for estimation of citicoline sodium and methyl paraben as shown in (**Table 3**).

Table 3: Optimisation of mobile phase

Sr. No.	Mobile Phase Composition	Retention Time (min)		Remark
		CITI	MetP	
1	Acetonitrile : HPLC grade water (20:80) of pH 2.8	2.2	4.626	Broadening of peak
2	0.1 M monobasic potassium phosphate : Acetonitrile (50:50)	1.982	5.784	Tailing, show bifurcation and bifurcate peaks developed.
3	0.1 M monobasic potassium phosphate : Acetonitrile (70: 30)	1.892	3.155	Peaks were not separated; peaks were not sharp and tailing.
4	0.1 M monobasic potassium phosphate : Acetonitrile (70: 30)	2.066	14.687	Sharp & well resolved peak with reproducible retention time

Optimization of flow rate and wavelength

For the optimization of flow rate and wavelength, we selected three flow rates of 0.5,1.0,102 ml/min and two wavengths of 272nm, 294nm. We optimized the flow rates and wavelengths individually by injecting the volume of 10 µl of standard citicoline sodium solution and methyl paraben solution in the RP-HPLC system with the run time of 20 minutes and Merck C_8 column and the respective ratio of mobile phase was 70:30 and obtained the graphs and optimized conditions shown in (**Table 4**).

Table 4: Optimisation of flow rate and wavelength

Sr. No.	Variable (Flow rate and Wavelength)	Retention Time (min)		Remark
		CITI	MetP	
1	0.5ml/min λ = 272nm	2.852	18.627	Tailing, broadening of peak
2	1.0ml/min λ = 294nm	2.624	16.265	Tailing, broadening of peak
3	1.5ml/min λ = 294nm	2.066	14.687	Sharp & well resolved peak with reproducible retention time

Optimization of column

For the optimization of column, we selected two columns of Merck C_8 column and Merck C_{18} column. We optimized the columns individually by injecting the volume of 10 µl of the standard citicoline sodium and methyl paraben solution in the RP- HPLC system with the flow rate of 1.5 ml/min and run time was 20 minutes. The respective ratio of mobile phase was 70: 30 at the wavelength of 294nm and obtained the graphs and optimized conditions shown in (**Table 5**).

Table 5: Optimisation of column

Sr. No.	Variable (Column)	Retention Time (min)		Remark
		CITI	MetP	
1	Merck C_{18}	2.852	16.845	Tailing, broadening of peak, peaks were not sharp
2	Merck C_8	2.066	14.687	Sharp & well resolved peak with reproducible retention time

METHOD VALIDATION

Method validation was carried out under the International Conference on Harmonization (ICH)

Guidelines for validation of analytical procedures[15]. The assay was validated with respect to linearity, precision, accuracy and sensitivity.

Linearity

The mobile phase was allowed to equilibrate with stationary phase. Each of the standard solution was injected separately. Linearity was evaluated by determining five standard working solutions in triplicate for HPLC. The aliquot portion of stock solution of CITI and MetP was diluted with mobile phase to get concentration of 80-100ppm and 8-10ppm. The chromatograms were recorded. The graph is plotted as conc. Vs peak area (Fig.5 A, B).

Precision

System Precision

The system precision was checked by using standard chemical substance to ensure that the analytical system is working properly. The retention time and area response of five determinations should be measured and calculate the relative standard deviation.

Method Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day and different analysts) and was expressed as relative standard deviation (R.S.D.). Repeatability was determined by performing three determinations on the same day at different time intervals and on three different days for interday precision.

Accuracy/recovery

In this study, accuracy was determined based on the recovery (percentage) of known amounts of standard CITI and MetP added in the assay samples. This was performed by analyzing CITI and MetP at three concentration levels (80, 100 and 120%). Samples were prepared in triplicate. The accuracy of the assay was determined by comparing the found concentration with the added concentration.

Sensitivity

Sensitivity of the method was determined by means of the detection limit (LOD) and quantification limit (LOQ). The LOD and LOQ were measured based on the method described by the International Conference on Harmonization. Calculations for LOD and LOQ were based on the standard deviation of the calibration curve (σ) and the slope of curve (S), using the equation $LOD=3.3\times\sigma/S$ and the equation $LOQ= 10\times\sigma/S$.

RESULTS AND DISCUSSION

Result of method development by RP-HPLC

A Reversed phase high performance liquid chromatography (RP-HPLC) method was developed for estimation of citicoline sodium and methyl paraben. In present study an attempt was made to modify experimental conditions in order to estimate drug (**Table 2**).

System suitability parameters

System suitability testing is an integral part of analytical procedures for estimation of pharmaceuticals. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability parameters established in present study are shown in (**Table 6**).

Linearity and Range

The standard calibration curve was linear over the concentration range 80-120 ppm for CITI and 8-12 ppm for MetP. The correlation coefficient obtained after linear regression analysis was 0.999 and 0.999 for CITI and MetP respectively. The equation of the calibration curve of CITI concentration is y= 13224+1E+06 and MetP concentration is y=20085x+10573 as shown in (**Fig.2 A, B**).

Precision

System precision

The results of system precision are shown in (**Table 7**). The R.S.D. value for CITI and MetP were found to be 0.11% and 0.04% respectively.

Method precision

The R.S.D. of repeatability (intra-day Table 8) are 0.05% for CITI and 0.18% for MetP and intermediate precision (inter-day Table 9 and different analysts Table 10) ranged between 0.09% and 0.06% and 0.09% and 0.08% for CITI and MetP respectively. These values show a low variability

between the values obtained for each concentration.

Accuracy/recovery

The results of the accuracy studies are shown in Table 11. The result showed that the R.S.D. was in the range of 0.45% and 0.53% for CITI and 0.49% and 0.92% for MetP that is less than 1%. The values obtained show a suitable accuracy for the analytical method.

Sensitivity

LOD and LOQ for CITI were found to be 0.0002µg/ml and 0.0008µg/ml and for MetP were found to be 0.0001µg/ml and 0.0005µg/ml, respectively. These values are adequate for the detection and quantification of CITI and MetP.

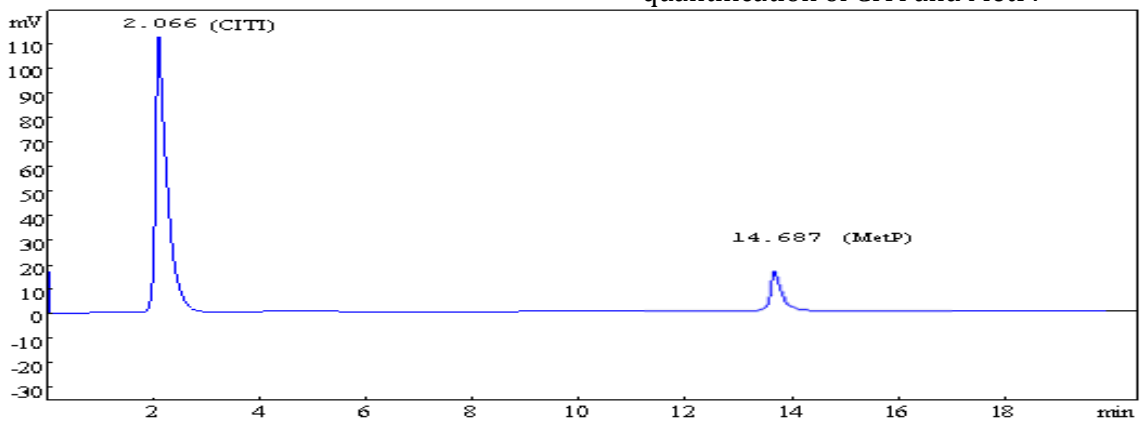


Figure3: Chromatogram of Standard Solution

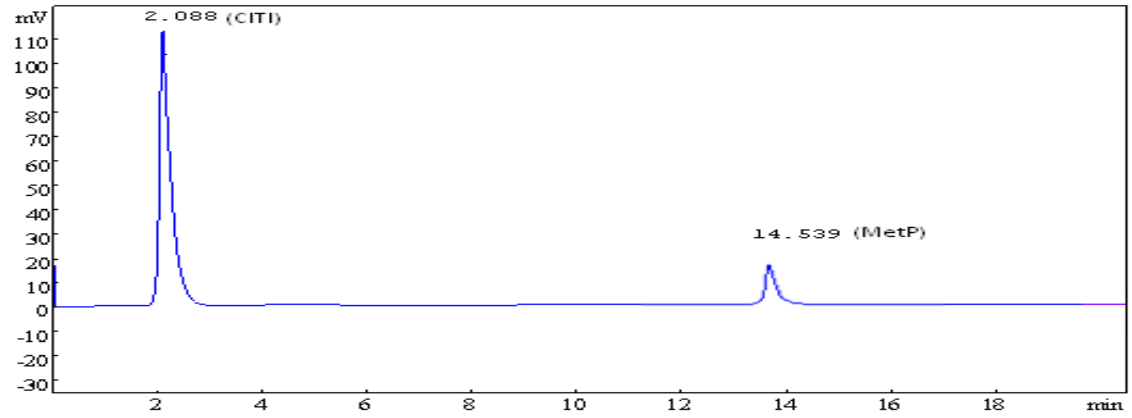
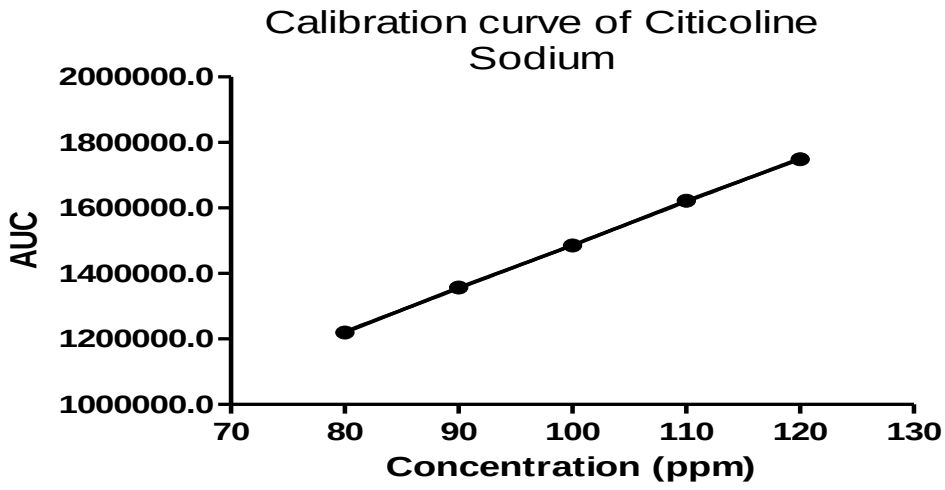


Figure 4: Chromatogram of Test Solution



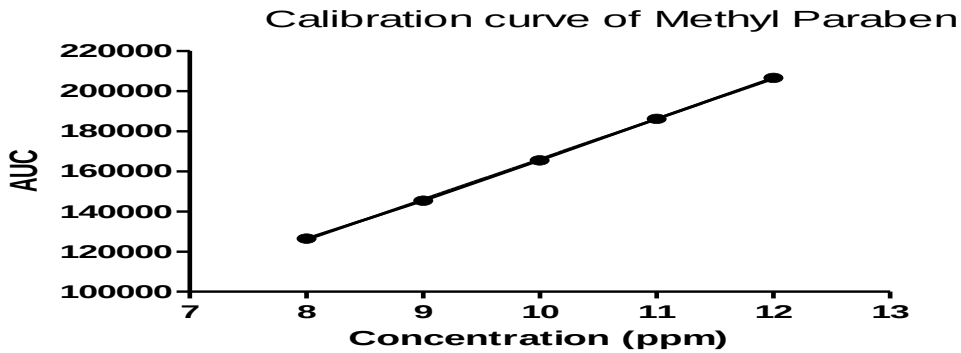


Figure 5 (A,B): Standard calibration curve for CITI and MetP

Table 6: Data of system suitability parameters

Peak area		Height(µV)		Theoretical plate		Retention time		Tailing factor	
CITI	**MetP**	**CITI**	**MetP**	**CITI**	**MetP**	**CITI**	**MetP**	**CITI**	**MetP**
15885	17622	18980	32192	4573	5631	2.085	14.231	1.388	1.15

Table 7: Data of System Precision

Sr. No.	**CITI**		**MetP**	
	Retention Time	**Area Response**	**Retention Time**	**Area Response**
1	2.08	1596043	14.64	186823
2	2.03	1595761	14.42	186914
3	2.10	1598143	14.67	186734
4	2.05	1594213	14.56	186871
5	2.07	1593475	14.32	186726
Mean	2.06	1595527	14.52	186843
± S.D.	0.027	1809.82	0.148	82.88
R.S.D. (%)	1.31	0.1134	1.01	0.044362

Table 8: Data of intraday study

Time	**Amount of oral drop taken (ml)**	**Amount of drugs estimated (mg/ml)**		**% Drug estimated**	
		CITI	**MetP**	**CITI**	**MetP**
0 hr	1.0	100.42	10.02	100.42	100.23
3 hr	1.0	100.36	10.01	100.36	100.19
6 hr	1.0	100.31	10.00	100.31	100.01

Statistics

Drug	**Mean (%)**	**± S.D.**	**R.S.D. (%)**
CITI	100.36	0.0550	0.0548
MetP	100.14	0.1175	0.1173

Table 9: Data of interday study

Days	**Amount of oral drop taken (ml)**	**Amount of drugs estimated (mg/ml)**		**% Drug estimated**	
		CITI	**MetP**	**CITI**	**MetP**
Day 1	1.0	99.69	9.96	99.69	99.68
Day 2	1.0	99.60	9.95	99.60	99.57
Day 3	1.0	99.50	9.95	99.50	99.51

Statistics

Drug	**Mean (%)**	**± S.D.**	**R.S.D. (%)**
CITI	99.59	0.0910	0.0913
MetP	99.59	0.0862	0.0866

Table 10: Data of different analyst

Analyst	Amount of oral drop taken (ml)	Amount of drugs estimated (mg/ml)		% Drug estimated	
		CITI	MetP	CITI	MetP
1	1.0	101.18	10.07	101.18	100.72
2	1.0	101.12	10.05	101.12	100.56
3	1.0	101.24	10.06	101.24	100.60
Statistics					
Drug	**Mean (%)**	**± S.D.**		**R.S.D. (%)**	
CITI	101.18	0.0600		0.0593	
MetP	100.62	0.0818		0.0812	

Table 11: Data of recovery study

S. N.	Accuracy level	Amount of pure drug added(µg)		Drug estimated (µg)		% Recovery	
		CITI	MetP	CITI	MetP	CITI	MetP
1.		8	0.8	7.98	0.789	99.76	98.63
2.	80%	8	0.8	7.98	0.794	99.76	99.35
3.		8	0.8	7.91	0.796	98.97	99.57
4.		10	1.0	9.95	0.990	99.52	99.07
5.	100%	10	1.0	9.99	0.990	99.96	99.58
6.		10	1.0	10.05	1.000	100.53	100.87
7.		12	1.2	11.94	1.190	99.55	99.57
8.	120%	12	1.2	11.89	1.190	99.12	99.18
9.		12	1.2	12.02	1.200	100.18	100.14
Statistics							
Drug	**Accuracy level**	**Mean (%)**		**±SD**		**R.S.D. (%)**	
CITI		99.49		0.4561		0.4584	
MetP	80%	99.18		0.4917		0.4954	
CITI		100.00		0.0506		0.5064	
MetP	100%	99.84		0.9277		0.9291	
CITI		99.61		0.5289		0.5309	
MetP	120%	99.63		0.4828		0.4845	

CONCLUSION

In the present research work to achieve highest precision in quantitative chromatography of CITI in pharmaceutical dosage form, a reverse phase liquid chromatography method for simultaneous estimation of CITI and MetP was developed and validated. The method was validated in terms of linearity, precision, accuracy, detection limit, quantification limit. It involves a simple procedure for the preparation of the samples and shorter run times for analytical procedure (less than 20 min). A low percent of organic solvent (Methanol 30%) was used in the composition of the mobile phase. Hence the present HPLC method can be considered simple, rapid, suitable and easy to apply for routine simultaneous estimation of Citicoline sodium and methyl paraben in liquid oral drop formulation.

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REFERENCES

1. Amol RJ, Bonde GC, Jadhav RT. Formulation and Evaluation of Citicoline Sustained Release Tablest. J. Pharma. Science 2011;3(1): 911-917.
2. O' Neil, M.J. The merck Index an Encyclopaedia of chemical, drugs and Biologicals, 14th ed., MERCK and Co. Inc. USA, 2006; 2319.
3. Sweetman, Martindale SC the complete Drug Reference, 35th ed., Pharmaceutical press, Londan UK, 2007; 2072.
4. Secades JJ, Lorenzo JL. Citicoline Pharmacological and Clinical Review: Methods Exp Clin Pharmacol 2003; 28: 1-56.
5. Saada B, Md. Baria F, Muhammad Saleha I, Ahmadb K, Mohd. Khairuddin Mohd. Talib. Simultaneous determination of preservatives (benzoic acid, sorbic acid, methylparaben and

propylparaben) in foodstuffs using high-performance liquid chromatography. Journal of Chromatography A 1073(2005); 393-397.
6. Kimbahune R, Surani S, Kabra P, Urmila GH. Spectrophotometric determination of Citicoline Sodium in pure form and Pharmaceutical Formulation. Scholars Research Library, Der Pharmacia Lettre 2010; 2(5): 353-357.
7. Patel JA, Panigrahi B, Patel CN, Ramalingan B, Modh K. Development and validation of a UV spectrophotometric method for estimation of Citicoline sodium in Bulk and Dosage form. Journal of Pharmacy Research December 2010; Vol.3. Issue: 2876-2878.
8. Malipatil SM, Patil SK, Deepthi M, Jahan K. New spectrophotometric methods for the determination of citicoline in pharmaceutical formulations. International Journal of Pharma Research and Development October 2010; Vol.2 Issue: 27-30.
9. Patil PM, Wankhede SB, Chaudhari PD. Simultaneous determination of Ketoprofen and Methyl Paraben, Propyl Paraben in Bulk and Formulated Gel by Spectrophotometry. Journal of Pharmaceutical and Scientific innovation Nov- Dec 2013; 2(6): 22-28.
10. Babu G, Prasad K, Kolla T, Vijayabaskaran M, Latha ST. Method development and validation of RP-HPLC for simultaneous estimation of Citicoline and Piracetam in Tablet dosage form. International Journal of Pharm Tech Research July-Sept 2011; Vol.3: 1311-1313.
11. Shah N, Pancholi SS. Development and Validation of Stability indicating RP- LC method for simultaneous estimation of Citicoline Sodium and Piracetam in bulk and Pharmaceutical formulation. International Journal of Pharmacy Research and Technology 2013; Vol. 3: 11-15.
12. Surani S, Kabra P, Kimbahune R, Sunil K, Nargund LVG. A Reverse Phase Liquid Chromatography Analysis of Citicoline Sodium in Pharmaceutical Dosage Form using Internal standard Method. Internal Standard Method. International Journal of Pharm Tech research; Vol.4, No.3: 1136-1141.
13. Abdollahpour A, Forouhi M, Shamsipur M and Yamini Y. High Performance Liquid Chromatographic determination of Sodium Benzoate, Methyl Paraben and Propyl Paraben as preservative components in Nystatin Suspension. Journal of the Iranian Chemical Society June 2010; Vol. 7, No. 2: 516-520.
14. Boonleang J. Tanthana C. Simultaneous Stability- indicating HPLC method for the determination of Cisapride, Methyl Paraben and Propyl Paraben in oral suspension. Songklanakarin Journal of Science and technology Aug.2010; 32(4): 379-385.
15. International Conference on Harmonization (ICH). Harmonized Tripartite Guidelines on 'Validation of Analytical procedure: Methodology', Operational from June 1997, The European products, Human Medicines Evaluation Unit.