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Research Article

STABILITY INDICATING REVERSED PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF RELATED SUBSTANCES OF CITICOLINE AND PIRACETAM IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: A high-performance liquid chromatography (HPLC) method was developed and validated to determine stability indicating method of Piracetam and Citicoline in a tablet dosage.

Methods: The separation was made using Inertsil C18, 250 × 4.6 mm, 5 µm column, mobile phase used contained phosphate buffer and acetonitrile in the gradient mode at wavelength of 205 nm for Piracetam and 280 nm for Citicoline on a PDA detector.

Results: The method showed good linearity for, respectively related substances of Citicoline and Piracetam with correlation coefficients in the range of 0.29-623 µg/mL and 0.48-1030 µg/mL, respectively. Method accuracy was assessed at three levels; the recovery ranged between 100.0% and 102% for Citicoline and for Piracetam between 94.3% and 109.1%. Limit of detection and quantification for Citicoline was 0.07 µg/mL - 0.25 µg/ml and for Piracetam 0.12 µg/mL - 0.41 µg/ml. The solution was found to be stable within 27 hrs at room temperature.

Conclusion: The method was demonstrated to be robust and simple, and suitable for industrial application for determination of related substances in the pharmaceutical preparation.

Keywords: Piracetam, Citicoline, Assay, Reversed phase-high performance liquid chromatography.

INTRODUCTION

Citicoline (Cytidine-5'-diphosphocholine) is intermediate in the biosynthesis of phosphatidylcholine. It plays an important role in cellular metabolism. Citicoline is readily absorbed in the gastrointestinal tract and widely distributed throughout the body, crosses the central nervous system (CNS), where it is incorporated into the membrane and microsomal fraction. It activates biosynthesis of structural phospholipids, increases brain metabolism, and acts on the levels of different neurotransmitters. It has a variety of applications in CNS injury models neurological disorders of the brain such as stroke, brain, trauma, Alzheimer's and Parkinson's disease [1-3].

Piracetam is a nootropic drug in the racetams group, with chemical name 2-oxo-1-pyrrolidine acetamide. Piracetam is a cyclic derivative of GABA

Both drugs are psychotherapeutic agents used as a psycho stimulant, nootropic, and neurotonics. These drugs increase the cerebral metabolism and level of various neurotransmitters including acetylcholine and dopamine. They exert their action by activating the biosynthesis of structural phospholipids in neuronal membrane.

The review of the literature regarding the quantitative analysis of Citicoline and Piracetam revealed that the attempts were made to develop analytical methods for estimation of Citicoline and Piracetam by spectrometric methods and LC methods the estimation individually [4-8]. The chemical structure of citicoline and Piracetam is shown in (Figs. 1 and 2).

UV spectrophotometric methods [9,10] and high-performance liquid chromatography (HPLC) methods [11-16] for simultaneous estimation as a combination of Citicoline and Piracetamin various dosage forms are also reported.

The objective of this analytical method development was to develop, optimize, and validate a rapid, specific, and economic and stability indicating reversed phase-HPLC (RP-HPLC) method for the simultaneous estimation of Citicoline and Piracetam in tablet dosage form on gradient mode offering better resolution of peak of interest as well as impurities so that it is useful even for studying degradation impurities when compared to isocratic mode analytical methods.

METHODS

Materials

HPLC: Waters HPLC 2 2695 series consisting pump, auto sampler, UV-visible detector (PDA), Thermostat column compartment connected with Waters [alliance] Empower2 software Balance Sartorius Cpa225d Semi Micro Balance Sonicator Bio-Technics India, Mumbai.

Reagents buffer reagents such as potassium dihydrogen phosphate, ion pairing reagents such as hexane sulfonic acid sodium salt. Organic solvents such as methanol and acetonitrile.

Methodology

Preparation of mobile phase buffer

6.8 g of potassium dihydrogen orthophosphate and 1.0 g of hexane sulfonic acid sodium salt dissolved in 1000 mL Mili-Q water, pH adjusted to 3.0±0.05 with diluted ortho phosphoric acid, filtered through 0.45 µm membrane filter and sonicated to degas.

Mobile Phase A: Filtered and degassed buffer.
Mobile Phase B: Buffer and methanol in the ratio 90:10 [% v/v].

Preparation of diluent: Mixture of buffer and acetonitrile in the ratio 90:10 [% v/v].

Chromatographic parameters
Column: Inertsil C18, (250 mm × 4.6 mm), 5 µm or equivalent
Detector: UV-visible (Use HPLC with PDA, MWD or DWD)
Detection Wavelength: 205 nm for Piracetam and 280 nm for Citicoline
Flow rate: 1.0 ml/minute
Injection volume: 10 µl
Column oven temperature: 30°C
Runtime: 45 minutes

The detection, i.e., 205 nm for Piracetam and 280 nm for Citicoline wavelength was selected based on UV spectrum scan.

Gradient program

Time (min)	Mobile Phase A	Mobile Phase B
0	100	0
8	100	0
26	10	90
35	90	10
40	100	0
45	100	0

Blank
Mixture of buffer and acetonitrile in the ratio 90:10 [% v/v].

Standard preparation
40 mg of Piracetam working standard and 27.2 mg of Citicoline sodium working standard (equivalent to 25 mg of Citicoline) was weighed into a clean and dry 100 ml volumetric flask, 50 ml of diluent was added and sonicated to dissolve, and then volume was made up to the mark with diluent. Further dilute 1 ml of above solution was diluted to 100 mL with diluent. (Concentration of Citicoline was about 2.5 µg/ml, and Piracetam was about 4.0 µg/ml).

Preparation of test solution
20 tablets were accurately weighed, and average weight was determined. The tablets were crushed to yield fine powder. The powder equivalent to 100 mg of Citicoline and 160 mg of Piracetam was weighed into a 200 ml volumetric flask. 100 mL of diluent was added and sonicated for 15 minutes with intermittent shaking. The solution was allowed to cool at room temperature. Diluted to the volume with diluent and mix. The solution was filtered through 0.45 µm nylon syringe filter (concentration of Citicoline was about 500 µg/ml, and Piracetam was about 800 µg/ml).

Placebo preparation
The placebo equivalent to 100 mg of Citicoline was weighed (~160 mg of Piracetam) into a 200 ml volumetric flask and 100 mL of diluent and sonicated for 15 minutes with intermittent shaking. The solution was allowed to cool at room temperature. Diluted to the volume with diluent and mix. The solution was filtered through 0.45 µm nylon syringe filter

Validation of quantitative HPLC method [17,18]
The optimized RP-HPLC method was validated according to the procedures described in International Conference on Harmonization (ICH) guidelines Q2 [R1] for the validation of analytical method (ICH 2005).

Specificity
Specificity was the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Linearity and range
It is the ability of the method to elicit test result that is directly proportional to analyte concentration within a given range.

Acceptance criteria: The correlation coefficient should be NLT 0.999.

Calibration curve was plotted over a concentration range of 0.29-623 µg/ml of Citicoline and 0.48-1030 µg/mL of Piracetam. Samples are prepared, filtered, and injected into HPLC system. Aliquots (10 µl) of each solution were injected from auto sampler under the operating chromatographic conditions described above. Calibration curve was constructed by plotting peak area verses concentration and the regression equation was calculated.

Accuracy and recovery
The absolute recovery of analytical method was measured as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard which has not been subjected to sample pretreatment and indicates whether the method provides a response for the entire amount of analyte that was present in the sample.

The accuracy of the method was determined by calculating recoveries of Citicoline and Piracetam by the standard addition method. Known amounts of standard solutions of Citicoline and Piracetam (0.05% to 1.00 % level) added to placebo preparation each in triplicate.

Limit of detection (LOD) and limit of quantification (LOQ)
The LOD and the LOQ of Citicoline and Piracetam were derived by calculating the signal-to-noise (S/N) ratio using the following equations designated by ICH guidelines.

$$LOD = 3.3 \times s/S$$

$$LOQ = 10 \times s/S$$

Where, s = the standard deviation of the response and S = slope of the calibration curve.

The LOD and LOQ were estimated by injecting lower concentration solutions of Citicoline and Piracetam and determining % relative standard deviation (RSD) of area responses of 6 replicate injections.

The LOD and LOQ were confirmed and recorded separately on the basis of the S/N ratio.

Method precision
The precision of the method was checked and verified by repeatability, inter-day precision, and variability due to the analyst. Repeatability was checked by injecting six test preparations into the HPLC system as per the test method. Ruggedness of the proposed analytical method was evaluated for variability studies such as variability due to analyst and variability due to a different day. Six test preparations were prepared and injected into the HPLC system as per the test method. The % of individual impurities for each sample was calculated. % RSD of individual impurities and total impurities was recorded.

Solution stability
The solution stability of Citicoline and Piracetam tablet was carried out by leaving both the test and standard solution in tightly capped volumetric flask at room temperature for 27 hrs. The same sample solution was analyzed initially and at various time intervals up to the 27 hrs throughout the study period. Both solutions were prepared in diluent.

Robustness
Robustness of the method was determined by small deliberate changes in flow rate (±10%), mobile phase composition (±2% absolute inorganic phase), pH (±0.2 unit), column oven temperature (±5°C), and detection wavelength (±5 nm). Placebo, diluted standard, and sample solution were injected under each of the robustness conditions and system suitability parameters were evaluated.

System precision
Diluted standard solution of Citicoline and Piracetam was prepared as per the proposed test procedure for repeatability studies. Six replicate injections were injected into the HPLC system. % RSD for the peak responses as the peak area was calculated.

Forced degradation study
Citicoline sodium API and Piracetam API were subjected to various degradation conditions, namely acid, base, peroxide, and thermal conditions. The peak purity of analyte peaks and impurity peaks was checked using Waters Empower 2 Software.

Forced degradation study of Citicoline and Piracetam

Acidic degradation

About 50 mg was accurately weighed and transferred to a 100 ml volumetric flask, 50 ml of diluent was added and sonicated to dissolve. 5 ml of 0.1 N HCl was added and kept at a 100°C for 3 hrs in water bath. The solution was allowed to attain room temperature. Then, the solution was neutralized by 0.1 N NaOH and diluted to volume with diluent. Filtered and injected.

Base degradation

About 50 mg of was accurately weighed and transferred to a 100 ml volumetric flask, 50 ml of diluent was added and sonicated to dissolve. 5 ml of 0.1 N NaOH was added and kept at a 100°C for 3 hrs in water bath. The solution was allowed to attain room temperature. Then, the solution was neutralized by 0.1 N HCl and diluted to volume with diluent. Filtered and injected.

Peroxide degradation

About 50 mg was accurately weighed and transferred to a 100 ml volumetric flask, 50 ml of diluent was added and sonicated to dissolve. 5 ml of 3% H_2O_2 was added and kept at a 100°C for 3 hrs in water bath. The solution was allowed to attain room temperature. Then, the solution diluted to volume with diluent filter and inject.

Thermal degradation

About 50 mg was accurately weighed and transferred to a 100 ml volumetric flask, 50 ml of diluent was added and sonicated to dissolve. 5 ml of 5 ml of diluent was added and kept at a 100°C for 3 hrs in water bath. The solution was allowed to attain room temperature. Then, the solution diluted to volume with diluent filter and inject.

Hydrolytic degradation

Weighed accurately powdered sample equivalent to 100 mg of Citicoline (~160 mg of Piracetam) into a 200 ml volumetric flask, added to it 20 ml of water and sonicated for 15 minutes with intermittent shaking. Heated the solution at 100°C for 3 hrs in water bath. The solution was allowed to attain room temperature. Then, the solution was diluted to volume with diluent Filter and inject.

The % degradation with respect to control sample and peak purity data for Citicoline and Piracetam under various degradation conditions has been given in Table 1.

RESULTS AND DISCUSSION

The optimized RP-HPLC method for related substances was validated according to the procedures described in ICH guidelines Q2 (R1) for the validation of analytical method (ICH 2005).

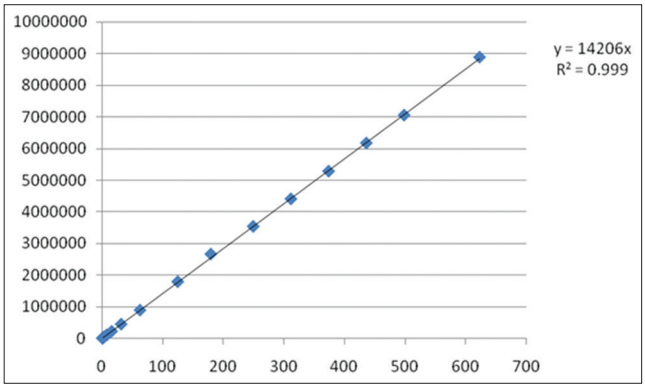
Specificity
Blank (diluent), placebo, diluted standard, and samples were injected into HPLC and peak Purity of analyte peaks and impurity peaks was checked. The impurity peaks were found to be well resolved from each other and from analytes peaks. No interference was observed at the retention time (RT) of analyte peak and impurity peak from the blank and placebo solution. The peaks were found to be pure and homogenous indicating stability indicating the ability of the proposed method.

Linearity and range
The linearity data for Piracetam and Citicoline has been given Graphs 1 and 2.

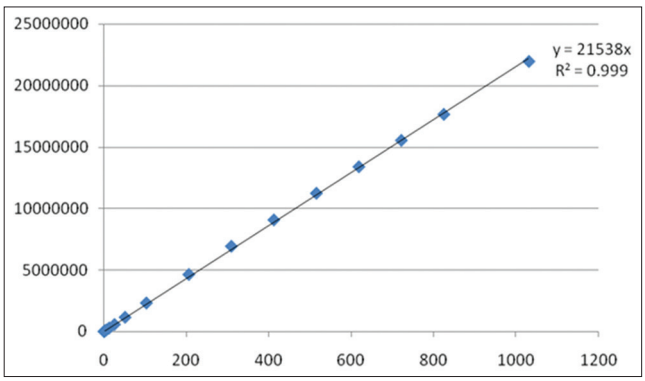
Table 1: Validation parameters and their acceptance criteria

S. No.	Parameters	Acceptance criteria
1	Specificity	No interference observed for response due to analyte or impurities of interest
2	Linearity and range	correlation coefficient NLT 0.999
3	Accuracy and recovery	NLT 98.0% and NMT 102.0% at various recovery levels
4	LOD	To be determined by test
5	LOQ	To be determined by test
6	Method precision	RSD NMT 2.0%
7	Solution stability	To be determined by test
9	System precision	RSD NMT 2.0%

SD: Standard deviation, RSD: Relative standard deviation, LOQ: Limit of quantification, LOD: Limit of detection



Graph 1: Linearity data for Citicoline



Graph 2: Linearity data for piracetam

The correlation coefficient was found NLT 0.98 that meets the acceptance criteria indicates that the responses are linear. This concludes that the method is linear throughout the range selected.

Accuracy
In table 2 and table 3 the data proved that The % recovery was found well within the accepted criteria (NLT 70.0% and NMT 130.0% at LOQ level and NLT 80.0% and NMT 120.0% at 0.1%, 0.3%, 0.5%, and 1.0% levels). Hence, the method is accurate throughout the selected range.

LOD and LOQ
The LOD and LOQ were estimated by injecting lower concentration solutions of Citicoline and Piracetam and determining % RSD of area responses of 6 replicate injections. Data obtained shown in Tables 4 and 5.

The % RSD for both the analytes was found well within the accepted criteria (NMT 10.0% at LOQ level and NMT 33.0% at LOD level). Hence, the method has a suitable level of LOD/LOQ.

Table 2: Accuracy data for Piracetam

Recovery level	% Recovery	Average recovery	% RSD
0.05% level	108.95	106.6	2.49
	103.72		
	107.11		
0.1% level	102.75	105.6	2.32
	106.87		
	107.11		
0.3% level	96.77	94.3	2.39
	92.41		
	93.58		
0.5% level	103.98	104.1	0.29
	104.43		
	103.86		
1.0% level	109.63	109.1	0.46
	108.78		
	108.74		

RSD: Relative standard deviation

Table 3: Accuracy data for Citicoline

Recovery level	% recovery	Average recovery	% RSD
LOQ level	102.89	102.1	5.44
	96.22		
	107.26		
0.1% level	101.62	104.3	2.58
	107.01		
	104.25		
0.3% level	101.51	101.1	1.01
	99.98		
	101.92		
0.5% level	101.21	101.3	0.09
	101.39		
	101.34		
1.0% level	100.75	100.9	0.18
	101.1		
	100.84		

RSD: Relative standard deviation, LOQ: Limit of quantification

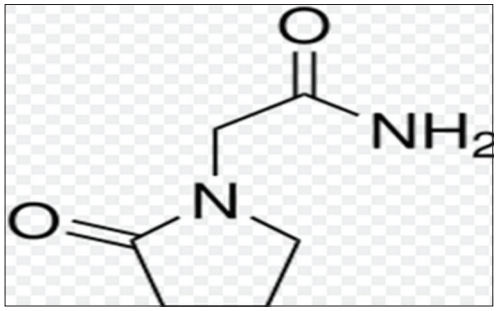


Fig. 1: Chemical structure of Piracetam

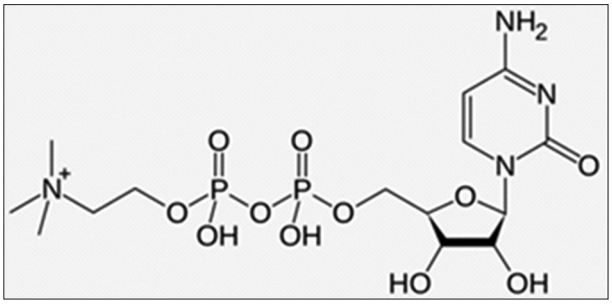


Fig. 2: Chemical structure of Citicoline

Table 4: LOD and LOQ data of Piracetam

Piracetam at 205 nm	Concentration	
	µg/ml	% w/w
LOD	0.12	0.02
LOQ	0.41	0.05
Injection no.	**Area counts**	
	LOD	**LOQ**
1	4593	22784
2	5379	23782
3	5287	23652
4	6043	23653
5	4892	23273
6	6041	23149
Mean	5373	23382
SD	590.3	382.1
RSD [%]	10.99	1.63

SD: Standard deviation, RSD: Relative standard deviation, LOQ: Limit of quantification, LOD: Limit of detection

Table 5: LOD and LOQ data of Citicoline

Citicoline at 280 nm	Concentration	
	µg/ml	% w/w
LOD	0.07	0.01
LOQ	0.25	0.05
Injection no.	**Area counts**	
	LOD	**LOQ**
1	5415	7468
2	4812	6855
3	5254	6806
4	5475	7006
5	5560	6879
6	5310	6687
Mean	5304	6950
SD	265.2	274.0
RSD (%)	5.00	3.94

SD: Standard deviation, RSD: Relative standard deviation, LOQ: Limit of quantification, LOD: Limit of detection

Solution stability

The data shown in Table 6 indicate that the sample solution is stable for 13 hrs at room temperature.

From the results, it is concluded that the solutions are stable at room temperature for about 27 hrs.

Robustness

System suitability parameters were met under all robustness conditions except a change in organic content in a gradient program where significant RT shift was observed for both the analyzes peaks. Hence, it is recommended that organic content should be strictly adhered to as per the method.

The system suitability data under robustness conditions has been summarized in Table 8.

The RT and area data under robustness conditions in comparison to control sample has been summarized in Table 8.

Forced degradation study

The developed RP-HPLC method for determination of related substances of Citicoline and Piracetam in Citicoline and Piracetam

Table 6: Solution stability data of Citicoline and Piracetam at room temperature

Time-point	Citicoline		Citicoline impurity		Piracetam	
	Area counts	Absolute % difference w.r.t. initial	Area counts	Absolute % difference w.r.t. initial	Area counts	Absolute % difference w.r.t. initial
Initial	7191962	-	5415	-	16125118	-
4.5 hr	7150525	0.58	5532	2.16	16060262	0.40
9 hr	7168792	0.32	5496	1.50	16145022	0.12
18 hr	7159497	0.45	5500	1.57	16072626	0.33
27 hr	7153346	0.54	5454	0.72	16101095	0.15
Acceptance criteria	NMT 2.0% for two consecutive time-points		NMT 10.0% for two consecutive time-points		NMT 2.0% for two consecutive time-points	

Table 7: Robustness data of Citicoline and Piracetam diluted standard

Parameters	Retention time		USP plate count		USP tailing factor	
	Citicoline	Piracetam	Citicoline	Piracetam	Citicoline	Piracetam
Control (IA)	4.278	12.403	5290	10725	1.04	1.03
Flow plus (1.1 ml/min)	3.956	11.101	4784	9480	1.05	1.03
Flow minus (0.9 ml/min)	4.804	13.546	6080	16725	1.06	1.28
[+] Organic content in Mobile Phase B (Buffer: MeOH::88:12)	4.298	12.412	5284	10851	1.03	0.90
[-] Organic content in Mobile Phase B (Buffer: MeOH::92:8)*	2.746	4.600	2675	2743	1.11	0.99
pH plus (pH=3.20)	4.149	11.532	5120	10201	1.07	1.01
pH minus (pH=2.80)	4.343	11.585	5378	9747	1.10	0.98
Temperature plus (35°C)	4.017	11.661	5525	10455	1.06	1.03
Temperature minus (25°C)	4.640	13.312	5457	11435	1.02	1.00
Wavelength plus	4.285 (λ=285)	12.469 (λ=215)	5281	10922	1.07	0.98
Wavelength minus	4.285 (λ=275)	12.471 (λ=205)	5293	10698	1.08	0.98

*Non-robust condition

Table 8: Robustness data for RT and area of Citicoline and Piracetam of sample

Parameters	RT		Area	
	Citicoline	Piracetam	Citicoline	Piracetam
Control (IA)	4.286	12.388	7142440	15789056
Flow Plus (1.1 ml/min)	3.919	11.101	6675779	14690096
Flow Minus (0.9 ml/minute)	4.770	13.722	8187676	18073171
[+] organic content in mobile Phase B (Buffer: MeOH::88:12)	4.297	12.329	7365848	16205575
[-] organic content in mobile Phase B (Buffer: MeOH::92:8)*	2.753	4.612	7231700	15470900
pH plus (pH=3.20)	4.094	11.529	7267393	16028965
pH minus (pH=2.80)	4.270	11.678	7435505	16099604
Temperature plus (35°C)	4.007	11.584	7162981	15912794
Temperature minus (25°C)	4.636	13.172	7247683	16057772
Wavelength plus	4.286	12.388	6538487	9550165
Wavelength minus	4.286	12.388	6922181	23407288

*Non-robust condition, RT: Retention time

Table 9: Peak purity data of Citicoline and Piracetam in degraded tablet sample

Degradation condition	Citicoline			Piracetam		
	% degradation	Purity angle	Purity threshold	% degradation	Purity angle	Purity threshold
Control sample	-	0.020	1.016	-	0.637	1.046
Acid degradation	3.6	0.023	1.012	5.1	0.111	1.039
Alkali degradation	2.4	0.039	1.023	16.7	0.127	1.069
Peroxide degradation	4.1	0.046	1.010	5.0	0.054	1.021
Thermal degradation	-	0.021	1.011	-	0.096	1.036
Hydrolytic degradation	2.9	0.025	1.019	4.0	0.046	1.032

combination tablet was found to be specific, accurate, and stability indicating and can be used for routine sample analysis. The retention time for acid degradation impurities in Citicoline & piracetam was found to be 3.59,5.37,6.01,6.79 mins and 10.06min, respectively.

The retention time for base degradation impurities in Citicoline & piracetam was found to be 3.55,5.35,6.00 mins and 17.85 min, respectively.

The retention time for peroxide degradation impurities in Citicoline & piracetam is 3.63,5.36,5.99 mins and 10.02min,respectively.The retention time for thermal degradation impurities in Citicoline & piracetam is 4.28 mins and 9.10,10.00min,respectively.The peak purity data is shown in (Table 9).

The retention time for citicoline and piracetam were 4.28 and 12.38 mins respectively.

CONCLUSION

A stability indicating RP-HPLC method has been developed and validated to determine related substances of Piracetam and Citicoline in a pharmaceutical dosage form.

The validation characteristics included accuracy, precision, specificity, linearity, and robust, and stability indicating. The method was demonstrated to be robust, resistant to small variations of chromatographic variables. The proposed HPLC gradient method is very well demonstrating the resolution of main peaks and impurity peaks of both citicoline and Piracetam. This method is fast, simple, can be used for determination of related substances in the pharmaceutical preparation. Future research is recommended in any other pharmaceutical dosage form as the method is optimized to elute the maximum numbers of impurities.

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