Research Article

DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE QUANTIFICATION OF CITICOLINE AND PIRACETAM

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ABSTRACT

The present work describes a reversed phase high performance liquid chromatographic method for simultaneous estimation of Citicoline and Piracetam in tablet dosage form. The estimation was carried out on a C18 column using a mixture of acetonitrile and 10 mM disodium hydrogen phosphate buffer in the ratio of 10:90 % v/v as a mobile phase. The pH of aqueous phase was adjusted to 6.0 with 1 % o-phosphoric acid. The flow rate of mobile phase was maintained at 1.0 mL/min. To achieve highest precision in the analysis, Paracetamol was used as an internal standard. All analytes were detected by measuring the absorbance at 210 nm. Total run time was 10 min; Citicoline, Piracetam and Paracetamol were eluted at the retention times of 2.6, 3.5 and 5.6 min respectively. Calibration curves were found linear over the concentration ranges of 10-100 µg/mL for Citicoline and 16-160 µg/mL for Piracetam. The method was validated for accuracy, precision, linearity, specificity and sensitivity as per ICH norms. From the validation study it was found that the method is specific, rapid, accurate and precise.

Keywords: Citicoline, Piracetam, RP-HPLC, Validation.

INTRODUCTION

Citicoline Sodium (CT), a psychostimulant, is chemically cytidine-5'-{trihydrogendiphosphate} p'-[2-{trimethyl ammonio}ethyl] ester inner salt while Piracetam (PM) is 2-oxo-1-pyrrolidine acetamide used as neurotonic. Both drugs are psychotherapeutic agents, used as psycho stimulant, nootropic and neurotonics. These drugs increase cerebral metabolism and level of various neurotransmitters, including acetylcholine and dopamine, exerting its action by activating the biosynthesis of structural phospholipids in neuronal membrane. These drugs increase the blood flow and oxygen consumption in brain.

Literature survey revealed that there are several methods such as Spectrophotometric[1-4], HPLC[5-11], LC-MS/Ms[12], Micellar Electro Kinetics Chromatography[13] and FTIR[14] reported for the analysis of CT and PM either as an individual drug in pure, pharmaceutical forms or in combination with impurities as well as in biological fluids. But, there is no HPLC method with internal standard reported for quantitative estimation of CT and PM in tablet dosage form using small proportion of organic solvent in mobile phase composition. Hence in the present work attempts have been made for the development and validation of simple, rapid, sensitive and precise HPLC method, using Paracetamol (PCM) as an internal standard (IS).

MATERIALS AND METHODS

Reagents and Chemicals

All the reagents like o- phosphoric acid, acetonitrile (Qualigens fine chemicals, Mumbai) and water used were HPLC grade. CT standard was obtained from M/S Strides Acrolab ltd, Bangalore while PM standard was supplied from Micro labs ltd. The marketed formulation NUTAM PLUS (Piracetam 800 mg, Citicoline 500 mg) was purchased from the local pharmacy.

Instrumentation

The HPLC system used was Shimadzu LC-20AT pump, Rheodyne injector (20µL), SPD-20A UV detector and the system was controlled through Spinchrom CFR software (version 2.1.4.93). Analytical column used for this method was Gracesmart C18 (250 mm x 4.6 mm, 5µm). Sartorius digital balance, Digisun electronics digital pH meter 7007, RC Systems sonicator and vacuum pump were used throughout the experiment.

Chromatographic Conditions

The composition of the mobile phase used was acetonitrile: 10 mM disodium hydrogen phosphate buffer (10:90% v/v) (pH of aqueous phase was adjusted to 6.0 with 1% o-phosphoric acid). The mobile phase was vacuum-filtered through 0.2 µm Supor 200 membrane and degassed by ultrasonication for 10 min before use. The mobile phase flow rate was set at 1.0 mL/min. All the standards and assay samples were filtered through 0.45 µm Supor 200 membrane 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 10 min. The absorbances of the eluents were monitored at 210 nm at a detection sensitivity of 0.1000 aufs. PCM (10 µg/mL) was used as an internal standard.

Standards and Sample Solutions Preparation

The mixed standard stock solutions of CT and PM were prepared by dissolving 12.5 mg of CT and 20 mg of PM in

25 mL of HPLC grade water to get concentration of 500 µg/mL of CT and 800 µg/mL of PM. Standard stock solution of PCM (IS) was prepared by dissolving accurately weighed 10 mg in 100 mL of HPLC grade water. From these resulting solutions, further serial dilutions were prepared in mobile phase for constructing calibration curves.

For sample solution preparation, 20 tablets of CT and PM (NUTAM PLUS Piracetam 800 mg, Citicoline 500 mg) were weighed and crushed to obtain fine powder. An accurately weighed tablet powder equivalent to about 12.5 mg of CT (20 mg of PM) was transferred to the 25 mL volumetric flask. 10 mL of HPLC grade water was added and sonicated for 10 min. The volume was made up to the mark with HPLC grade water to get concentration of 500 µg/mL of CT and 800 µg/mL of PM and filtered through whatman filter paper no 41. From the above solution 1 mL was transferred into 10 mL volumetric flask along with 1 mL of PCM solution (100 µg/mL) and made up to mark with mobile phase (50 µg/mL CT, 80 µg/mL PM and 10 µg/mL PCM). Similarly from the standard stock solution (500 µg/mL of CT and 800 µg/mL of PM), 1mL was transferred to 10 mL volumetric flask along with 1 mL of IS (PCM 100 µg/mL). The volume was made up to the mark with mobile phase to get the concentration of 50 µg/mL CT, 80 µg/mL PM and 10 µg/mL PCM.

Both of these solutions (standard and sample) were filtered through 0.45 µm Supor 200 membrane filter using syringe before injection. After equilibration of column with the mobile phase indicated by a stable baseline, aliquots of sample (20µL) were injected. The chromatograms were observed for the peak area (Fig 1). The amount of CT and PM present in the tablets were calculated using single point analysis method and results are shown in table 1.

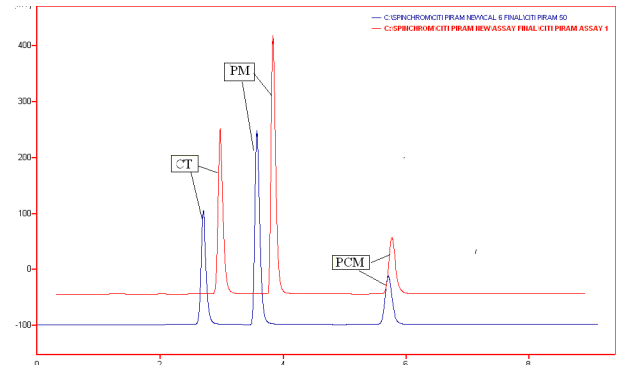

Figure 1: Overlain (3D view) chromatogram of sample and standard solution of CT 50 µg/mL and PM 80 µg/mL in acetonitrile:10 mM disodium hydrogen phosphate buffer (10:90 % v/v, pH 6) at flow rate of 1mL/min at 210 nm using C18 column.

Table 1: Results of assay of Nutam Plus tablet

Component	Label claim (mg)	Mean amount found (mg) n=6	Mean % Assay ± RSD
CT	500.00	539.56	107.90 ± 0.5819
PM	800.00	789.62	98.70 ± 0.5061

Method Validation

Method validation was carried in accordance to the International Conference on Harmonization (ICH) guidelines for validation of analytical procedures[15]. The assay was validated with respect to linearity, precision, accuracy, sensitivity and robustness.

Accuracy/Recovery

Accuracy of the developed method was confirmed by performing a recovery study as per ICH norms at three different concentration levels (80%, 100%, 120%) by replicate analysis (n = 3). Standard drugs were added to a preanalyzed sample solution and chromatograms were recorded. The percent of standard drugs recovered were calculated.

Precision

The precision of the method was determined by repeatability, intermediate precision (intra-day, inter-day) and was expressed as % relative standard deviation (%R.S.D.). Intra- day precision was determined by performing analysis of triplicate injections of three different concentrations of combination on the same day at different time intervals and on three different days for inter-day precision.

Linearity

Calibration curves were obtained from injecting the six sets of nine serial dilutions of mixed standard stock solution of CT and PM (1.25:2, 2.5:4, 5:8, 10:16, 15:24, 20:32, 25:40, 50:80 and 100:160 µg/mL) with 10 µg/mL of IS. The linearity was determined for CT and PM separately by plotting a calibration graph of ratio of peak area of drug to IS against their respective concentration.

Sensitivity

Sensitivity of the method was determined by means of the detection limit (LOD) and quantification limit (LOQ). Calculations for LOD and LOQ were based on the standard deviation of the Y-intercepts of the six calibration curves (σ) and the average slope of the six calibration curve (*S*), using the equation LOD= 3.3×σ/*S* and the equation LOQ= 10×σ/*S*.

Robustness

Robustness of the method was evaluated by the analysis of solution under varying experimental conditions such as pH of the mobile phase and flow rate. The flow rate was varied ±0.02 mL/ min (2%) and pH of the mobile phase was changed ±0.12 units (2%). Their effects on the retention time (*t*R), tailing factor (*T*) and resolution of the peaks (*R*) were studied.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

The chromatographic conditions were adjusted to provide the best performance of the assay. For system optimization the important parameters such as type and

concentration of organic solvents, pH and mobile phase flow rate were investigated.

Effect of Mobile Phase Composition

Different proportions mobile phases like water: acetonitrile (50:50, 70:30 %v/v), water: methanol (70:30, 80:20, 90:10 %v/v pH 2.5), water: acetonitrile (70:30, 80:20,90:10 %v/v pH 2.8), 10mM Potassium dihydrogen phosphate buffer: acetonitrile (90:10 %v/v pH2.5, 3.0 with o-phosphoric acid and pH 6.0 with triethylamine) and 10mM disodium hydrogen phosphate buffer: acetonitrile (90:10 %v/v, pH 6.0,6.5 with o-phosphoric acid) were tested. As 90:10 %v/v proportion of 10mM disodium hydrogen phosphate buffer: acetonitrile at pH 6.0 with o-phosphoric acid showed better resolution(R) among CT, PM and PCM with good peak symmetry and maximum number of theoretical plates, hence this composition of the mobile phase was finalised.

Internal Standard

Caffeine and Paracetamol were tested as an IS for the developed chromatographic procedure. Among them, Paracetamol (PCM) eluted before 10 min of the analysis and has a better symmetry and resolution with respect to CT and PM. Therefore, PCM has been chosen as an IS.

Method Validation

Accuracy

Method accuracy was checked by standard addition method and percentage recovery and percentage relative standard deviation were calculated. The results obtained (Table 2) indicate that recoveries were good, not less than 98% and percentage relative standard deviations were less than 2%.

Table 2: Results of Recovery studies

Component	Concentration added (µg/ml)	Concentration recovered (µg/ml)	Recovery (%)	R.S.D. (%) (*n*=3)
CT	40.00	40.79	102.37	0.5235
	50.00	51.14	102.58	0.4028
	60.00	61.02	101.95	0.7948
PM	64.00	64.09	100.17	0.6496
	80.00	80.39	100.51	0.3793
	96.00	95.96	99.98	0.7950

Precision

Three different concentrations of combination of CT and PM were selected for intra-day and inter-day precision. The % RSD of the study was found to be less than 2% as shown in table 3.

Linearity

The linearity of this method was found to be in the concentration ranges 10-100 µg/ml for CT and 16-160µg/ml for PM. Y=0.037x + 0.063 and Y = 0.038x + 0.022 are linear regression equations with correlation coefficients of 0.999 for CT and PM respectively and were determined from linearity curve.

Table 3: Results of Precision studies

Component	Concentration (µg/ml)	R.S.D. (%), intra-day (n=3)	R.S.D. (%), inter-day (n=3)
CT	20.00	1.2395	1.5694
	50.00	1.4288	1.8368
	100.00	1.0845	1.9090
PM	32.00	1.0852	1.5108
	80.00	0.7493	1.8139
	160.00	0.7098	0.7621

Limits of Detection and Quantification

The limits of detection (LOD) and quantification (LOQ) were established by evaluating the minimum level at which the analyte could be readily detected and quantified with accuracy, respectively. The LOD was found to be 0.0379 µg/mL and 1.4857 µg/mL for CT and PM respectively and the LOQ was found to be 2.1328 µg/mL and 4.5022 µg/mL for CT and PM respectively.

System Suitability

System suitability was performed to confirm that the equipment was adequate for the analysis to be performed. The test was carried out by making six replicate injections of a standard solution containing 50µg/mL CT, 80 µg/mL PM and 10 µg/mL PCM (IS) and analyzing each solute for their peak area, theoretical plates (*N*), resolution (R) and tailing factor (T). The results of system suitability study in comparison with the required limits are shown in Table 4. The proposed method fulfils these requirements within the accepted limits.

Table 4: System suitability results of the proposed method

Analyte	R[a]	N[b]	T[c]	%RSD	
				Rt	Peak Area Ratio
CT	-	4663	1.44	1.76	0.8237
PM	5.78	9587	1.43	1.63	0.5153
IS	11.56	11228	1.31	2.00	-
Required limits	*R* >2	*N*>2000	*T* < 2	R.S.D. < 2%	

a- Resolution factor, b- Number of theoretical plates, c-Tailing factor, n=6

Robustness

During the robustness study, peak symmetry (T) was maintained and the retention times were not significantly changed as shown in Table 5. These facts suggest that the method did not change with time and experimental conditions.

Table 5: Results of Robustness of the Method (n=3)

Parameter	Analyte	pH			Flow rate (mL/min)		
		5.88	6.0	6.12	0.98	1.0	1.02
R[a]	*CT -PM*	5.73	5.78	5.77	5.82	5.78	5.70
	PM - PCM	11.37	11.56	11.10	11.72	11.56	11.47
T[b]	CT	1.40	1.44	1.43	1.49	1.44	1.40
	PM	1.41	1.43	1.45	1.47	1.43	1.38
Rt[c] (min)	CT	2.67	2.65	2.61	2.70	2.65	2.62
	PM	3.51	3.55	3.52	3.51	3.55	3.60

a- Resolution factor, b-Tailing Factor, c-Retention time

CONCLUSION

In the present research work to achieve highest precision in quantitative estimation of CT and PM from tablet dosage form, a reversed phase liquid chromatography method was developed and validated using PCM as an IS. The method was validated in terms of linearity, precision, accuracy, detection limit, quantification limit and robustness.

The developed method has a simple procedure for the preparation of the samples, shorter run time for chromatographic analysis (less than 10 min) and a low percent of organic solvent (acetonitrile 10%) in the composition of the mobile phase. Hence the proposed RP-HPLC method can be considered as simple, rapid, suitable and easy to apply for routine analysis of CT and PM in pharmaceutical dosage form.

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