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RESEARCH ARTICLE

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF A SIMULTANEOUS DETERMINATION OF CITICOLINE AND PIRACETAM AT SINGLE WAVELENGTH

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ABSTRACT

A simple, selective, rapid, precise, accurate, sensitive and robust RP-HPLC method has been developed and validated for the determination of citicoline and piracetam in tablet dosage form. The method was carried out on a column- Phenomenex Gemini C_{18} (250×4.6mm,5µ) with a mobile phase consisting of 0.02M potassium dihydrogen phosphate buffer and acetonitrile in the ratio 95:5 (pH adjusted to 6.9 with 0.1%v/v triethylamine). Detection was carried out at 220 nm and the flow rate is 1.0 ml/min. The retention time of citicoline and piracetam was found to be 2.3 and 2.8 respectively. Results of the analysis were validated statistically and by recovery studies; accuracy (99.3-102.9%) and reproducibility was found to be satisfactory. The developed method was validated in terms of accuracy, precision, linearity, and limit of detection, limit of quantification, ruggnedness and robustness studies. The proposed method can be successfully used to determine the drug content of marketed formulation.

Key words: RP-HPLC; Citicoline; Piracetam; Simultaneous determination; Method Validation.

INTRODUCTION:

Citicoline is chemically 5'O [hydroxyl ({hydroxyl [2(trimethylammonio) ethoxy] phosphoryl} oxy) phosphoryl] cytidine) [Fig-1a]. It is a psycho stimulant/nootropic drug [1].

Citicoline complex organic molecules that functions as an intermediate in the biosynthesis of cell membrane phospholipids. Citicoline is also known as CDP-choline and cytidine diphosphate choline. The basic structure of a nucleotide contain ribose with a nitrogenous base and a phosphate group. CDP-citicoline is composed of ribose, pyrophosphate cytosine (a nitrogenous base) and chloline. Piracetam is chemically 2-oxo-1-pyrrolidine acetamide [Fig-1b]. It is used as a nootropic drug [3]. It is a drug which is claimed to enchance cognition and memory, slow down brain aging. Increase blood flow and oxygen to be brain, aid stroke recovery and improve Alzhelmis.Down syndrome, dementia and dyslexia, among. According to the literature survey conducted, it was observed that no method was reported for the estimation of Citicoline and Piracetam in combined dosage form. Some methods for the estimation of Piracetam individual drug were carried out by HPLC methods [2, 4]. Some methods for the estimation of Citicoline individual drug were carried out by Spectrophotometry and HPLC in biological fluids and pharmaceutical formulations [5-9]. Our study attempt to develop an accurate, precise, specific, linear, simple, rapid, validated and cost effective analytical method for Citicoline and Piracetam in tablet dosage form by RP-HPLC method based on FDA guidance and the ICH guidelines [10-11].

2. EXPERIMENTAL:

2.1 Instrumentation and chromatographic conditions

The HPLC system, used for the method development and method validation was shimadzu (Japan) Model, equipped with LC-2010 HPLC pump, online degasser, column heater, SPD-20A UV detector, Rheodyne injector and Spinchrome software. Chromatographic separation was performed isocratically using a column was-Phenomenex Gemini C_{18} (250×4.6mm, 5µ) with a mobile phase of 0.02M potassium dihydrogen phosphate buffer pH 6.9 and acetonitrile in the ratio of (95:5, v/v).A membrane filter 0.45µm porosity was used to filter and degas the mobile phase. The flow rate was 1mL min-1 and the detector was set at 220 nm.The volume of the sample solution injected was 20µL.The analysis was carried out at ambient temperature.

2.2. *Materials and reagents*

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The gift samples of Citicoline and piracetam raw materials was procured from The Madras pharmaceuticals(Chennai,india).Analytical reagent grade potassium dihydrogen phosphate(KH_2PO_4) were procured from Qualigens fine chemicals(Mumbai, India), and HPLC grade acetonitrile (ACN) were procured from E-Merck(Mumbai, India). A Millipore Milli Q plus water purification system (Miford, USA, was used to prepare distilled water. Test samples, "Strolin P" composed of citicoline 500 mg and piracetam 800 mg per tablet, purchased from Torrent pharmaceuticals Ltd, Ahmedabad.

2.3 Solution preparation:

2.3.1. Preparation of buffer

2.72 gm potassium dihydrogen phosphate transferred to 1000ml volumetric flask and adds water to dissolve it completely and adjust the pH to 6.9 with 0.1v/v triethylamine.

2.3.2. CITICOLINE and PIRACETAM standard stock solution

Standard stock solutions were prepared by dissolving citicoline and piracetam in 100ml diluent (buffer: acetonitrile; 95:5 v/v) to obtain concentration of Citicoline (1250µg/mL-1) and piracetam (2000µg/mL-1).The standard stock solutions were suitably diluted with diluent to obtain the working standard solutions of both citicoline (125µg/mL-1) and piracetam (200 µg/mL-1).

2.3.3. CITICOLNE and PIRACETAM test stock solution

Twenty tablets (500 mg citicoline and 800 mg piracetam per tablet) were weighed and crushed to fine powder. An accurately weighed powder sample equivalent to 200 mg of piracetam was weighed, transferred to a 100ml volumetric flask and volume made up to 70 ml with diluent. The solution was sonicated for about 30 min, then diluted to volume with the same solvent and filtered through What man filter paper. Working samples solutions were freshly prepared by diluting suitable volumes of the stock sample solution with diluent.

3. METHOD DEVELOPMENT:

A variety of mobile phases were investigated in the development for the analysis of Citicoline and Piracetm tablet dosage form. The suitability of mobile phase was decided on the basis of selectivity and sensitivity of the assay and separation of drug.

4. METHOD VALIDATION:

The optimized chromatographic conditions were validated by evaluating specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness and ruggedness and system suitability parameters in accordance with the ICH guideline Q2 (R1).

4.1. Specificity

The specificity defined as the ability of method to measure the analyte accurately and specifically in the presence of tablet exicipients.

4.2. Linearity

Standard stock solution of the drug was diluted to prepare linearity standard solutions in the concentration range of 105-160 µg mL^{-1} for Citicoline and 160-240 µg mL^{-1} for Piracetam (80-120% of target level). Such solutions was prepared and analyzed to plot a calibration curve. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

4.3. Accuracy (Recovery Studies)

The accuracy of sample preparation was determined by recovery study, which was determined by spiking the sample with 80%, 100% and 120%. These mixtures were analyzed by the proposed method. The experiment was performed in triplicate and recovery (%), RSD (%) and standard error of mean (SEM) of spiked drugs were calculated.

4.4. Precision

The precision of the proposed method was evaluated by caring out six independent assays of test sample. RSD (%) of six assay values obtained was calculated to measure the repeatability of retention times and peak area of standard and sample.

4.5. Selectivity

Selectivity of the current method was demonstrated by good separation of the two analytes from each other. Fig.2. Furthermore, excipient of the tablet formulation did not interfere with the active ingredients of the drug product.

4.6. Limits of detection (LOD) and quantification (LOQ)

Limits of detection (LOD) and lower limit of quantification (LOQ) were estimated from the signal-to-noise ratio. The detection limit was determined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantification limit was determined as the lowest concentration level that provided a peak area with ten times signal-to-noise.

4.7. Robustness and Ruggedness

The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The conditions studied were flow rate (altered by ± 0.2mL min^{-1}), mobile phase composition (buffer pH altered ± 0.2,) and detection nm (±2). To test the ruggedness of the method, the analysis was done on different instruments on different days and with different chemists to check for any changes in the chromatograph and the percentage RSD was calculated.

4.8. System suitability

To know reproducibility of the method, system suitability test was employed to establish the parameters such as tailing factors, theoretical plates, repeatability and resolution.

5. RESULTS AND DISCUSSION:

5.1. Optimization of chromatographic conditions

The RP-HPLC Procedure was optimized with a view to developing a method for assay of citicoline and piracetam. No internal standard was used because no extraction or separation step was involved. The main objective of the chromatographic method is to separate citicoline and piracetam, using different stationary phases such as C_{18}, C_8 as well as with different mobile phases containing buffer like phosphate and acetate with different pH (4 to 8) and using organic modifiers like acetonitrile, methanol and ethanol in the mobile phase. The chromatographic conditions achieved on phenomenex-Gemeni C_{18} (250 x 4.6 mm) column with 5µ particle with the mobile phase consist of a mixture of 0.02M potassium dihydrogen phosphate buffer (pH adjusted to 6.9 using 0.1% v/v triethylamine) and acetonitrile (95:5, v/v). The flow rate was 1ml/min. Peak shape of the citicoline and piracetam were found to be symmetrical in this optimized chromatographic conditions and also citicoline and piracetam were separated with resolution greater than 2. Good separation is seen as the retention times of citicoline and piracetam were 2.3 and 2.8 min, respectively, using the chromatographic conditions described above. The results of the analysis of tablet formulations are reported in Table 3.

5.2 Method validation:

For precision study, %RSD of citicoline and piracetam. Shows that the chromatogram indicating no inte``rference between two drugs at 220 nm. Good separation is seen as the retention times of citicoline and piracetam were 2.3 and 2.8 min, respectively, using the chromatographic conditions described above. No interfering peaks were encountered in the blanks samples. Fig 2.The chromatographic run time 7min was sufficient for sample analysis that allows analyzing large number of samples in a short period of time. Injection of blank buffer into HPLC column represented that no peak could be seen on chromatogram. In this sample over line spectrum shows in Fig 3.

5.3 .Linearity and calibration

The calibration plot for the method was linear over the concentration range of 105-160µg mL-1 for citicoline and 160-240µg mL-1 for piracetam. The calibration equations were y = 23.861x+23.449 (r^2=0.9999) for citicoline and y = 18.787x-8.4144 (r2 =0.9999) for piracetam (Table 4 and Fig 4 &5).

5.4. Accuracy (Recovery studies)

The recovery studies were carried out at 80%, 100%, and 120% of the test concentration as per ICH guidelines. The percentage recovery of citicoline and piracetam at all the three levels was found to be satisfactory and the results obtained are listed in Table 2.

5.5. Precision

The precision of this method was evaluated by calculating the RSD of the peak area of six replicate injection of standard solution, which found to be 0.460% and 0.422% for citicoline and piracetam respectively (Table 7 & 8).

5.6. Selectivity

Selectivity of the current method was demonstrated by good separation of the two analytes from each other (Fig.2). Furthermore, excipient of the tablet formulation did not interfere with the active ingredients of the drug product.

5.7. Limit of detection and limit of quantification

The limit of detection and quantification decide about the sensitivity of the method and were calculated from the peak signal–to-noise ratios. In the present study, the LOQ values for citicoline and piracetam were 0.063 and 0.192 µg/ml and detection limits were found to be 0.200 and 0.607µg/ml respectively (Table 1).

5.8. Robustness and ruggedness

Robustness of the method was determined by making slight change in the chromatographic condition. The ruggedness of the method was estimated by carrying out the experiment as per proposed method in duplicates using different column and analyst on different days. The method was carried out on INERTSIL-ODS C_{18} and phenomenex-Gemeni C_{18} columns. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed was sufficiently robust for normal expected variations in chromatographic conditions (Table 5 & 6).

5.9. System suitability

System suitability test was employed to know reproducibility of the method and the parameters such as tailing factors, theoretical plates, repeatability and resolution are tabulated in Table 1.

Table 1: Analytical Parameters

Parameters	Citicoline	Piracetam
Linear dynamic range	80-120(µg/ml)	80-120(µg/ml)
Retention time	2.3	2.8
Resolution	2.0000	
Theoretical plates	2124	2416
Tailing Factor	1.33	1.25
Regression Coefficient	0.9999	0.9999
LOD (µg/mL)	0.063	0.200
LOQ (µg/mL)	0.192	0.607

Table 2: Recovery Studies

Recovery for citicoline and piracetam					
Concentration	Quantity added	Quantity recovered for citicoline	Quantity recovered for piracetam	% Recovery for citicoline*	% Recovery for piracetam*
80%	307.12	311.10	304.93	101.2	99.3
100%	383.9	393.11	379.29	102.4	98.8
120%	460.68	474.03	463.44	102.9	100.6
Mean				**102.2**	**99.6**
SD				**0.91**	**0.94**
% RSD				**0.89**	**0.94**

*Average of three determinations, mean ±standard deviation

Table 3: The Estimation of Citicoline Sodium and Piracetam in tablet dosage form

Content	% of Citicoline	% of Piracetam
Drug	**100.74±0.21**	**100.21±50**

*Average of three determinations, mean ±standard deviation

Table 4: Linearity for citicoline and piracetam

Levels	Citicoline Conc. (µg/ml)	Piracetam Conc. (µg/ml)
Level 1(80%)	105.92	159.44
Level 2(90%)	119.16	179.37
Level 3(100%)	132.40	199.30
Level 4(110%)	145.64	219.23
Level 5(120%)	158.88	239.16

Table 5: Ruggedness studies of Citicoline and Piracetam

Day 1	% of Citicoline	% of Piracetam
Analyst1,Inst 1	98.90	102.00
Analyst2,Inst 1	99.20	100.10
Analyst1,Inst 2	99.70	101.40
Analyst2,Inst 2	99.60	99.30
Day 2, Analyst 1,Inst 1	99.35	100.20
Analyst 2,Inst 1	99.72	99.26
Analyst 1,Inst 2	99.78	100.80
Analyst 2,Inst 2	99.50	90.90

Table 6: Robustness Studies of citicoline and piracetam in different pH, different nm and different flow rate

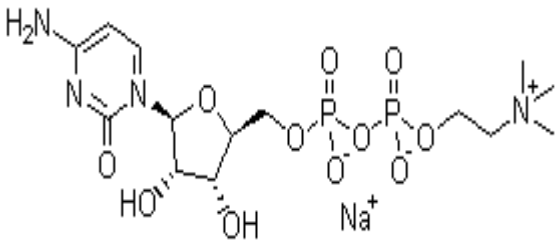
Citicoline				Piracetam		
Content	Avg area	SD	%RSD	Avg area	SD	%RSD
pH (-0.2)	2407.270	5.21	0.216	1825.0285	3.27	0.204
pH (+0.2)	2135.981	7.95	0.372	1581.3367	6.17	0.390
Nm(-2)	2022.314	4.14	0.205	1490.5354	3.53	0.236
Nm(+2)	2340.557	7.29	0.311	1741.7141	2.88	0.165
Flow rate(-0.2)	1655.129	10.30	1.31	1806.067	8.12	0.77
Flow rate(+0.2)	1516.636	11.21	1.43	1941.898	7.98	0.73

Table 7: Method Precision Studies

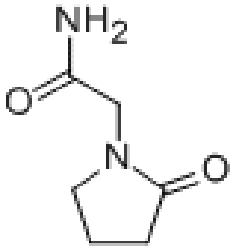
Method precision for citicoline and Piracetam	**% Assay for Citicoline**	**% Assay for Piracetam**
Sample Preparation-1	102.0	98.9
Sample Preparation-2	100.1	99.2
Sample Preparation-3	101.4	99.7
Sample Preparation-4	101.3	99.6
Sample Preparation-5	101.9	100.2
Sample Preparation-6	100.3	98.4
Avg	101.16	99.33
SD	0.79	0.63
%RSD	**0.79**	**0.64**

Table 8: System Precision Studies

System precision for citicoline and Piracetam	**Area of citicoline**	**Area of piracetam**
Standard Preparation-1	2250.5012	1759.2345
Standard Preparation-2	2240.2364	1748.2348
Standard pre paration-3	2267.2541	1750.2130
Standard Preparation-4	2239.2324	1758.1360
Standard preparation-5	2252.2310	1762.1280
Standard preparation-6	2257.2280	1768.0992
Avg	**2251.1138**	**1756.674**
SD	**10.57**	**7.42**
%RSD	**0.460**	**0.422**



(1a)



(1b)

Figure 1: Chemical structure for (1a) citicoline, 1(b) piracetam

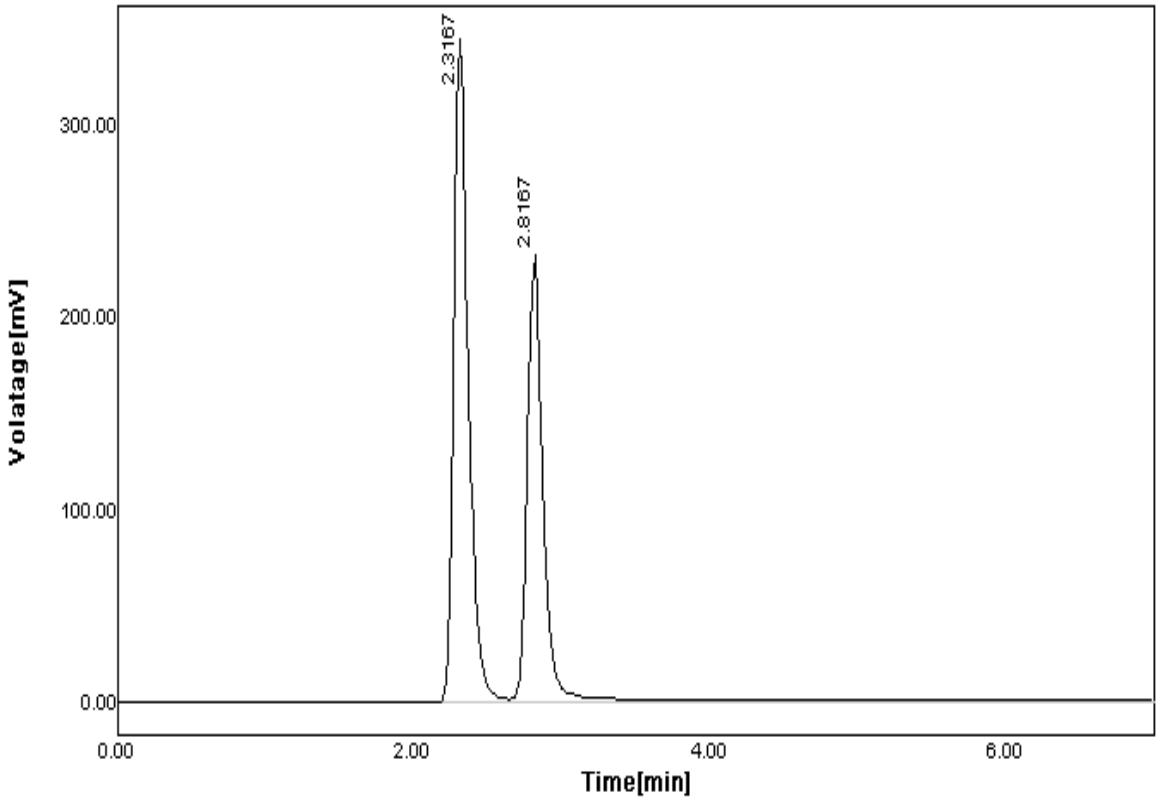


Figure 2: Typical Chromatogram of Citicoline and Piracetam

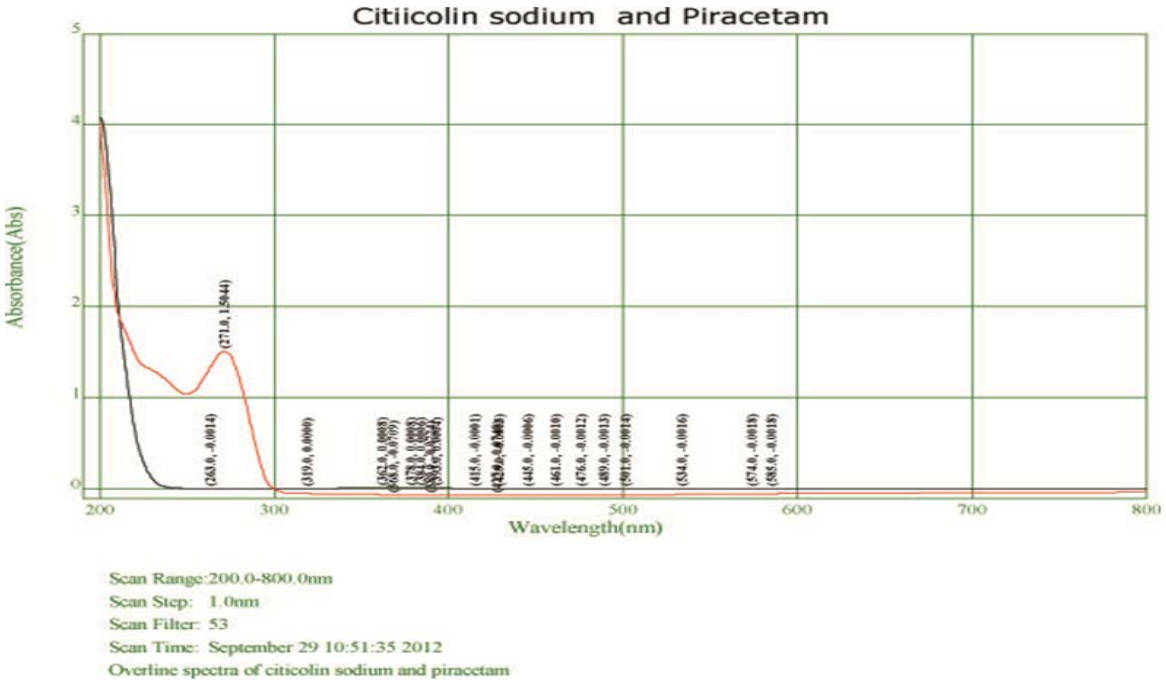


Figure 3: Over line Spectrum for citicoline and piracetam for nanometer detection

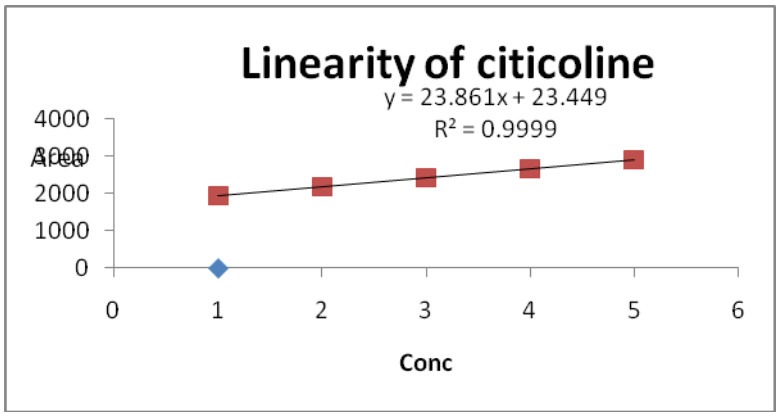


Figure 4: Linearity of Citicoline

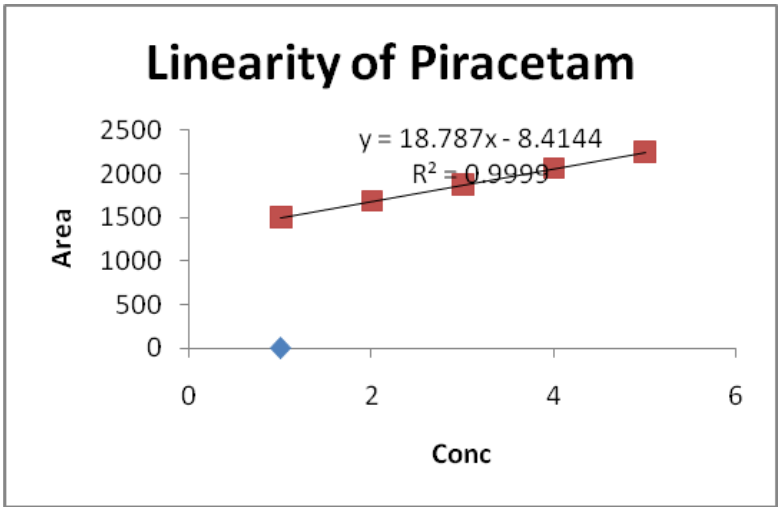


Figure 5: Linearity of Piracetam

6. CONCLUSION:

An HPLC method for simultaneous estimation of citicoline and piracetam for in single wavelength has not been reported. The presented method in addition to its novelty for determination of two ingredients at single wavelength is sufficiently rapid, simple and sensitive as well as precise and accurate for the accuracy and precision. The assay of the two active ingredient ingredients was not interfered in the expients in the product. The linearity, accuracy, precision, limit of detection and quantification, specificity-selectivity of the method and were established.

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