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SIMULTANEOUS ESTIMATION OF PIRACETAM AND CARBAMAZEPINE IN BULK AND PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC

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Abstract : RP-HPLC method has shown adequate simultaneous separation for a Piracetam & Carbamazepine . For the method development, several preliminary trial runs were taken on the pre-validated RP-HPLC system by varying the concentration of mobile and different solvent containing various ratios of acetonitrile, methanol, water and phosphate buffer pH 7.4 were tried for separation and resolution of peaks of both drugs to get the optimized parameters. Finally, chromatographic separation was carried out with Inertsil C18 (4.6 x 250mm, 5µm). The mobile phase used for isocratic elution was prepared by mixing phosphate buffer pH 7.4: methanol (50:50 v/v). Before use, the mobile phase was filtered through 0.45 µm membrane filter and degassed by ultra-sonication. The flow rate was 1.0 mL/min, column temperature ambient, the injection volume was 20 µl, and detection was performed at 260 nm using a UV detector. The retention time was obtained to be 3.51±0.15 min for piracetam and 4.82 ±0.21 min.
Keywords: RP-HPLC, chromatographic saperation, spectroscopy.

1. INTRODUCTION

Pharmaceutical analysis simply means analysis of pharmaceuticals. Webster' dictionary defines pharmaceutical as a medical drug. It is generally known that a pharmaceutical is a therapeutic interest. A more appropriate term for a pharmaceutical is active pharmaceutical ingredient (API) or active ingredient to distinguish it from a formulated product or drug product is prepared by formulating a drug substance with inert ingredient (excipient) to prepare a drug product that is suitable for administration to patients. It is well known in the pharmaceutical industry that pharmaceutical analysts in research and development (R&D) play a very comprehensive role in new drug development and follow up activities to ensure that a new drug product meets the established standards is stable and continue to approved by regulatory authorities ,assuring that all batches of drug product are made to the specific standards utilization of approved ingredients and production method becomes the responsibility of pharmaceutical analysts in the quality control (QC) or quality assurance department

The methods are generally developed in an analytical R&D department and transferred to QC or other departments as needed. At times theyare transferred to other divisions.y now it should be quite apparent that pharmaceutical analysts play a major role in assuring the identity, safety, efficacy, and quality of drug product safety and efficacy studies required that drug substance and drug product meet two critical requirements.

1. Established identity and purity

2. Established bio availability/dissolution

1.1 Scope and Significance of Pharmaceutical Analysis

Pharmaceutical companies rely upon both qualitative and quantitative chemical analysis to ensure that the raw material used meet all the desired specifications, and also to check the quality of the final product. The examination of raw material is carried out to ensure that there is no unusual substance present which might deteriorate the manufacturing process or appear as a harmful impurity in the final product. The quantity of required ingredient in raw material is determined by a procedure known as *Assay*. The final manufactured product is subjected to quality control to ensure that desired components are present within a range and impurities do not exceed certain specified limits [2]

Some specific use of analysis is under mentioned:

1) Quantitative analysis of air, water, soil samples to determine the level of pollution.
2) Chemical analysis to assist diagnosis of illness and monitoring the condition of patients.
3) In farming, nature of soil and level of fertilizer application is analyzed.
4) In geology, composition of the rock and soil is carried out.

1.2 Types of Analysis:

In general analysis is divided into two major parts:

Qualitative Analysis *(what substances are present in the given sample)*

Quantitative Analysis *(to determine the quantity of each component in the given sample)*

Quantitative:

Quantitative analysis seeks to establish the amount of a given element orcompound in a sample.

The factors which must be taken into account when selecting an appropriate method of analysis are:

The nature of the information sought

(A) The size of sample available and the proportion of the constituent to be determined

(B) The purpose for which the analytical data is required.

1.2 Introduction to Chromatography

The term chromatography 3(Greek kromatos –colour & graphos-written means colour writing .Mikhail Twestt (1906) - invented the chromatography. The IUPAC has defined chromatography as "a method used primarily for the separation of component of a sample, in which the component are distributed between two phases, one of which is stationary while the other moves .the stationary may be a solid or liquid supported on a solid or a gel and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid"

1.3 High Performance Liquid Chromatography (HPLC) [3-7]

High performance liquid chromatography (HPLC) is a process, which separates mixture containing two or more components under high pressure. in this the stationary phase is packed in a column one end of which is attached to a source of pressurized liquid mobile phase. High performance liquid chromatography is the fastest growing analytical technique for the analysis of drugs. Its simplicity, high specificity and wide range of sensitivity makes it ideal for the analysis of many drugs in both dosage forms and biological fluids.

HPLC is also known as high pressure liquid chromatography .It is essential form of column chromatography in which the stationary phase is consist of small particles (3-50μm) pickings contained in a column with a small pore(2-5mm) one end of which is attached to a source of pressurized liquid eluent (mobile phase). The three form of high performance liquid chromatography most often used are ion-exchange, partition and adsorption.

2. NEED OF THE STUDY.

Piracetam is chemically designed as 2-oxo-1- pyrrolidine acetamide. It is a nootropic, psycho pharmacological drug. Piracetam has important role in augmenting cognition and memory, impeding brain disaster, and improving blood and oxygen flow to the brain. It has been known to enhance mental conditions like dementia, dyslexia, and Alzheimer's disease and Down syndrome whereas, Carbamazepine is, 5-H-dibenze azepine-5-carboxamide, is a tricyclic lipophilic compound that is a first choice antiepileptic drug to control secondarily generalised tonic-clonic seizures and partial seizures .

Literature assessment disclosed that Piracetam has been estimated by various methods in pure and pharmaceutical dosage forms including RP-HPLC, UV also the single Carbamazepine method development and validation by UV and RP-

HPLC was done. But simultaneous determination of both drugs has not been reported yet by development and validation parameters that are included in the present research work

The present work is novel because it involves the newly analytical method to estimate the quantification of Piracetam and Carbamazepine simultaneously. So, there is need to develop cost effective, robust analytical validated method for estimation of Piracetam and Carbamazepine in bulk pharmaceutical dosage form.

3. REVIEW OF LITERATURE

Literature survey was carried out on the proposed topic by referring various scientific journals, online and offline also referred various text books available in college library. This survey reveals that no such articles were reported on the proposed work and some related articles.

4. AIM

Analytical Method Development and Validation for Quantitative Simultaneous Estimation of Piracetam and Carbamazepine in bulk and Pharmaceutical dosage form by Using RP-HPLC

5. OBJECTIVES

(1) To study the detailed literature survey on Piracetam and Carbamazepine drugs
(2) To developed simple, accurate, sensitive, reproducible validated HPLC method for Simultaneous estimation of Piracetam and Carbamazepine in bulk and Pharmaceutical dosage form by RP-HPLC.
(3) To validate the developed method as per ICH Q2 (R1) guidelines.
(4) Statistic comparison of the developed method with the literature method.

6. PLAN OF WORK

1. Literature Survey
2. Selection of drug molecules and Selection of formulation
3. Selection of analytical techniques
    a) UV-Spectrophotometric method
    b) Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method
4. Estimation of Acetazolamide by UV & RP-HPLC method involving following steps:
    a) Drug solubility
    b) Selection of wavelength
    c) Preparation of solution and Linearity study by UV Spectrophotometer
    d) Selection of stationary and mobile phase
    e) Preparation of solution
    f) Selection and optimization of chromatographic condition
5. Validation of UV & HPLC method as per ICH Guidelines
    I. Linearity and Range
    II. Precision
    III. Robustness
    IV. Ruggedness
    V. Specificity
    VI. Accuracy
    VII. Limit of detection (LOD)
    VIII. Limit of quantitation (LOQ)
    IX. System suitability
6. Compilation of data
7. Writing of Thesis, Publication on the performed research work

7. MATERIALS & METHOD

Table 1: List of Materials

Sr.No.	Name of Materials	Supplier

1	Piracetam	Varda Biotech Pvt. Ltd, Mumbai
2	Carbamazepine	Varda Biotech Pvt. Ltd, Mumbai
3	HPLC Grade Water	S D Fine-Chem Limited
4	Potassium Dihydrogen phosphate	Merck Life Science Pvt. Ltd
5	Methanol	Merck Life Science Pvt. Ltd
6	Di-potassium Hydrogen Phosphate	Themo Fisher Scientific Ind. Pvt. Ltd

Table 2: List of Equipment's

Sr.No.	Name of Equipments	Make	Model
1	Analytical Balance	SHIMANDZU	UniBloCAP
2	pH Meter	CONTECH	pH-103
3	Sonicator	DUEX Instrument	UC 179-02-04
4	Filter	Ecotest	NY 0.45µm
5	FT-IR	JASCO	FT/IR-4600
6	UV Spectrophotometer	SHIMANDZU	UV-1900i
7	Column	Chromasil 60-5-CN	150X4.6mm, 5µm
8	HPLC	SHIMANDZU	LC-2010 AHT

7.1 UV Method Development of Piracetam and Carbamazepine

7.1.1 Preliminary Studies and Spectral Studies of Piracetam and Carbamazepine

FT-IR Studies and UV Spectrometry studies of Piracetam and Carbamazepine

The FT-IR spectra for both the drugs were recorded by using FT–IR (Brukers Alpha) to confirm the identity of the drugs. Solubility of both the drugs was determined by dissolving the drugs in various solvents varying in their polarity.

Identification by IR Spectroscopy

Each 20 mg of Piracetam and Carbamazepine API and KBr was mixed properly then carefully triturated in a mortar pestle. Make thin plate, place in IR chamber and IR Spectrum was scanned.

7.1.2 Optimization of Uv Spectrometry Conditions and Method Development

Various Solvents like water, Methanol, ethanol and phosphate buffer were used for the optimization of diluents for the Uv method development of Piracetam and Carbamazepine. Optimized diluents were used for the preparation of standard solution and further dilutions.

7.1.3 Preparation of Piracetam and Carbamazepine Standard Solution

Standard solution was prepared by accurately weighed 50 mg of Piracetam and 50 mg of Carbamazepine working standard into a 50 ml volumetric flask, added 50 ml of Methanol, shake and sonicated to dissolve the content, made up the volume with methanol and filtered through 0.45 micron membrane filter. The solution was further diluted with water to obtain the required concentration of standard solution (10-60 µg/ml) for Piracetam and (8-28 µg/ml) for Carbamazepine respectively.

7.1.4 Determination of λ max (Selection of Wavelength)

The standard solution of Piracetam and Carbamazepine was scanned in the wavelength range of 200- 800 nm on a UV-Visible Spectrophotometer from this, wavelength corresponding to maximum absorbance (λ max) was found to be 206 nm for Piracetam and 284 nm for Carbamazepine respectively.

7.1.5 Development of standard curve for the Piracetam and Carbamazepine .

Various dilutions of Piracetam and Carbamazepine from the standard solutions were prepared for the Piracetam 10,20,30,40,50 and60 µg/ml were prepared whereas, for the Carbamazepine 8,12,16,20,24,28 µg/ml standards were prepared by using Methanol at the fixed wavelength 206 nm and 284 nm respectively.

7.2 UV Method Validation of Piracetam and Carbamazepine .

Developed UV method for estimation of Piracetam and Carbamazepine was validated as per ICH guideline for evaluating different parameters like Linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantification (LOQ).

7.2.1 Linearity

Linearity of Piracetam and Carbamazepine was established using estimation of absorbance of six different calibration standards and the calibration curve plot.

7.2.2 Accuracy

The solutions prepared i.e., 80%, 100% and 120% solutions were injected into the column. The amounts added and amounts estimated for Piracetam and Carbamazepine and the individual recovery and mean recovery values were calculated.
Following formula was used to calculate percent recovery.
% RC= [SPS-S/SP] × 100
Where, SPS= Amount found in the spiked sample
S= Amount found in the sample
SP= Amount added to the sample
% RC= Percent recovery

7.2.3 Precision

Intra- and inter-day precision of the method was established at three concentration levels. Intra-day precision was established by preparing nine different solutions of 10 µg/ml, 30µg/ml and 60 µg/ml for Piracetam and 8 µg/ml, 20 µg/ml, 28 µg/ml for Carbamazepine respectively and its analysis at morning, afternoon and evening time. Deviation in results in terms of % relative standard deviation (% RSD) was calculated. Inter-day precision of Piracetam and Carbamazepine was established by analyzing the above mentioned solutions at three consecutive days.

7.2.4 Robustness

Robustness of the method was evaluated by changing the solvents. Three different solvents viz. Ethanol, methanol and distilled water were used for dissolving Piracetam and Carbamazepine and the absorbance of each was determined. Piracetam and Carbamazepine levels in each sample were estimated using pre-defined calibration curve. Results were represented in terms of % RSD.

7.2.5 Ruggedness

Ruggedness of the method was determined by carrying out the analysis of Piracetam and Carbamazepine solutions (10, 30 and 60 µg/ml for Piracetam and 8,20,28 µg/ml for Carbamazepine) at three different (25°C, 37°C and 60°C) temperatures and absorbance were noted and % RSD was calculated.

7.2.6 Limit of Detection (LOD)

The LOD of the developed UV method for Piracetam and Carbamazepine was calculated using the following formula
LOD=3.3×SD/S
Where, SD= standard deviation of Y- intercepts
S=Slope

7.2.7 Limit of Quantitation (LOQ)

The LOQ of the developed UV method for Piracetam and Carbamazepine was calculated using following formula
LOQ=10×SD/S
Where, SD= standard deviation of Y- intercepts
S=Slope

7.3 Preparation of Mobile Phase:

Prepared a mixture of Water and methanol in the ratio of 60:40 v/v mixed well and degassed it.

7.3.1 Preparation of Standard Stock Solution:

Piracetam (10 mg) and Carbamazepine (20 mg) were accurately weighed and transferred into a 10 ml clean, dry volumetric flask and about 7ml of diluent was added and sonicated to dissolve the drugs completely and the volume was made up to the mark with the same solvent. (Stock solution I) Later 5ml of solution was pippetted out from the above stock solution into a 25 ml volumetric flask and the volume was made upto the mark with the diluent (stock solution II) Further 1.5 ml of solution was pippetted out from stock solution II into a 10ml volumetric flask and diluted to the mark with diluent (stock solution III)

7.3.2 Preparation of Sample Solution:

Tablet powder equivalent to 400 mg of Piracetam and 200 mg of Carbamazepine was accurately weighed and transferred into a 100 ml clean dry volumetric flask and about 70 ml of diluent was added and sonicated to dissolve the drugs completely and the volume was made up to the mark with the same solvent. (Stock solution I) Further 5 ml of solution was pippetted out from the above stock solution into a 25 ml volumetric flask and diluted up to the mark with diluent (Stock solution II) Further 1 ml of above solution was pippetted out from the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent (Stock solution III).

7.3.3 Optimization of Chromatographic Conditions and Method Development

Several chromatographic runs for mixture of Piracetam and Carbamazepine were taken in various combinations of mobile phase. Proper selection of the method depends upon the nature of the sample (ionic/ionizable/neutral molecule, its molecular weight and solubility). Here, the reverse phase HPLC method was selected for the initial separation owing to its simplicity, suitability, ruggedness and its wider usage. Various mobile phases such as Methanol and water (80:20), Methanol and water (60:40), were tried. Finally, Water and Methanol in the ratio of 50:50 was selected as mobile phase for further chromatographic study.

7.4 Method Validation

Validation study was intended to show that the method is suitable for assay and stability studies of Piracetam and Carbamazepine in Semisolid dosage form. The method validation was carried out as per ICH guidelines for specificity, forced degradation, precision, linearity, accuracy and stability in analytical solution (ICH 1996, Q2 (R1) ICH, 2005).

7.4.1 System Suitability Study

20 µl of standard preparations in five replicates previously prepared were injected. The chromatograms and the peak responses were measured for Piracetam and Carbamazepine. System suitability of the method was evaluated in terms of Retention time (RT), peak area, tailing factor, resolution and theoretical plate.

7.4.2 Specificity

Specificity is 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. Blank solution, individual standard solution and mixed standard solution of Piracetam (30 µg/ml) and Carbamazepine (20 µg/ml) were injected into the HPLC system. The peak purity data of Piracetam and Carbamazepine was compared there should not be any interference at the retention time of the main peaks.

7.4.2 Precision

a) System Precision

Six replicates of the mixed standard solution containing the 30 µg/ml of Piracetam and 20 µg/ml of Carbamazepine were injected into HPLC system. Prepared solutions were analyzed as per the proposed method. The mean, SD and % RSD were calculated.

b) Method Precision

Six samples containing the known amounts of Piracetam and Carbamazepine (30µg/ml &20µg/ml respectively) were analyzed as per test method and the % assay and % RSD for both the drugs was calculated.

c) Intraday and Inter-day Precision

The intraday precision of the assay method for Piracetam and Carbamazepine was evaluated at three concentration levels prepared from the sample stock solution (Piracetam 10, 20, 30 µg/ml & Carbamazepine 8, 12, 16µg/ml) by performing analysis at an interval of two hrs for 12 hrs. The inter- day precision study was also performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels as used for intraday study.

7.4.4 Accuracy (Recovery Study)

Accuracy study of pre-optimized method was calculated using recovery studies by performing the standard addition method. Three levels of percent i.e. 80, 100 and 120 % amount was added externally to the solutions with predefined amount of Piracetam (10, 30 and 60 µg/mL) and amount of Carbamazepine (8,20,28 µg/mL) and the % recovery was calculated.

% Recovery = A/B+C x 100
A = Total drug estimated (mg)
B = Wt. (mg) of drug contributed by tablet powder
C = Amount of pure drug added (mg)

7.4.5 Linearity and Range

Linearity for the Piracetam and Carbamazepine was determined by preparing the standard solutions at five concentrations in six replicates levels in the range of 10-60 µg/ml for Piracetam and 8-28 µg/ml for Carbamazepine from the stock solutions. 20 µl of each solution was injected into the HPLC system and the peak area of the chromatogram obtained was noted. The mean area with its standard deviation and % relative standard deviation of peak areas were calculated. Mean AUC was plotted against concentration to obtain the calibration curve. Regression equations, correlation coefficients were computed from calibration curves

7.4.6 Stability in Analytical Solution

Stability of Piracetam and Carbamazepine in analytical solution was verified by analyzing the sample (20 µg/ml and 40 µg/ml for Piracetam and 12 and 20 µg/ml Carbamazepine respectively) in six replicates before and after 24 hrs by storing

in refrigerator (8 °C) and at room condition. The % assay was calculated from the peak areas of Piracetam and Carbamazepine.

7.4.7 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ for Piracetam and Carbamazepine were calculated from slope and standard deviation of the response for Piracetam and Carbamazepine. The LOD and LOQ were determined using equations.

$LOD=3.3\times SD/S$ $LOQ=10\times SD/S$

Where; σ = Standard deviation of response,

S = Slope of calibration curve

7.4.8 Robustness

Pre-analyzed sample solution containing mixture of 30 µg/ml of Piracetam, 20 µg/ml of Carbamazepine was prepared and analyzed as per proposed method by changing the flow rate to 1.2 ml/min and 0.8 ml/min. The system suitability parameters and peak areas (or % assay) was evaluated in each condition and the results were compared with method precision results.

7.4.9 Ruggedness

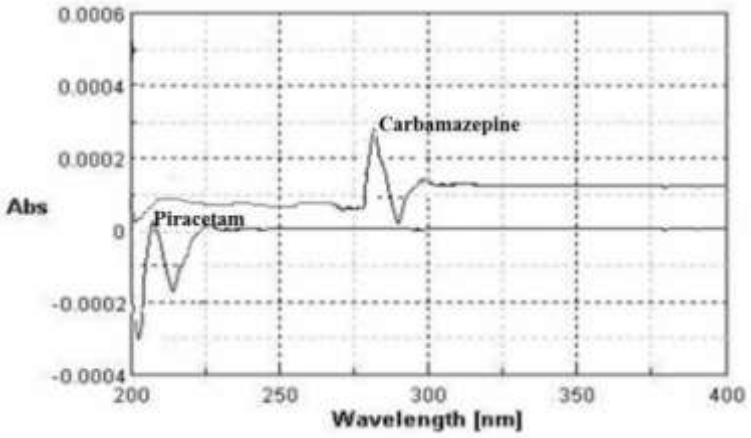
Ruggedness of the method was determined by carrying out the analysis of Piracetam (10, 20, 30 µg/ml) and Carbamazepine solutions (8, 16 and 24 µg/ml) at three different (25°C, 37°C and 60°C) temperatures and area were noted and % RSD was Calculated

8.0 Results and Discussion

8.1 Preliminary Studies and Spectral Studies of Piracetam and Carbamazepine

8.1.1 FT-IR Studies and UV Spectrometry studies of Piracetam and Carbamazepine

The preliminary identification was carried out by recording the FTIR spectrum for Piracetam and Carbamazepine. The observed group frequencies are tabulated in table. Piracetam is freely soluble in water so we took water as media for the Uv



and HPLC development of Piracetam whereas as Carbamazepine is soluble in methanol so, we took the Methanol as medium for the estimation of carbamazepine by Uv and HPLC. From the overlain spectrum Piracetam and Carbamazepine 215 nm was selected as wavelength for chromatographic method development.

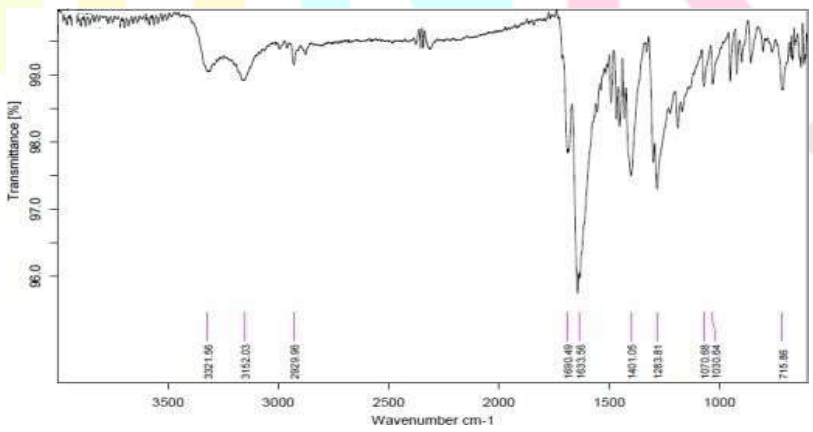


Table 3: Observed Group Frequencies by FT-IR

Name of Drug	Observed IR Frequencies	Functional group Present
Piracetam	1690.49	C=O stretching, primary amide
	1633.56	C=C stretching, conjugated alkene
Carbamazepine	3321.66	C-H stretching, alkyne
	3152.03	O-H stretching, alcohol
	2829.96	N-H stretching, amine salt
	1283.81	C-O stretching, alkyl aryl ether

8.1.2 Optimization of Uv Spectrometry Conditions and Method Development Piracetam:

Observations

It is found that Piracetam is freely soluble in water found to be stable in presence of water so for Uv method development used for dilutions.

For Carbamazepine: Observations

It is found that carbamazepine is soluble in methanol so decided to solubilizes carbamazepine in methanol and further diluted with water as diluent for the dilution.

8.1.3 Determination of λ max (Selection of Wavelength)

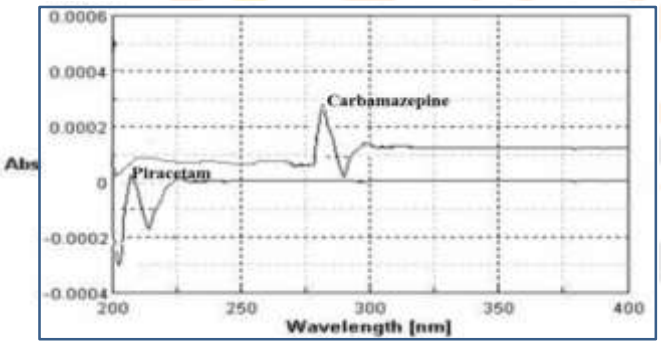


Figure 5: Absorbance maxima of Piracetam and Carbamazepine

Absorbance maxima of Piracetam and Carbamazepine were found to be on 206 nm and 284 nm respectively. The calibration curve of both the drugs was developed by using these maxima as fixed wavelength.

8.1.4 Development of standard curve for the Piracetam and Carbamazepine

8.1.4.1 Piracetam

The calibration curve of Piracetam was performed and graph plotted concentration vs. absorbance. The absorbance values of different concentration were noted. The regression equation was found to be y = 0.0143x+0.0996, with R^2 value of 0.9997. The graph was found to be linear.

Table 5: Concentration range and respective absorbance of Piracetam

Sr No.	Concentration (ppm)	Absorbance
1.	10	0.2361
2.	20	0.3887
3.	30	0.5338
4.	40	0.6737
5.	50	0.8139
6.	60	0.9537

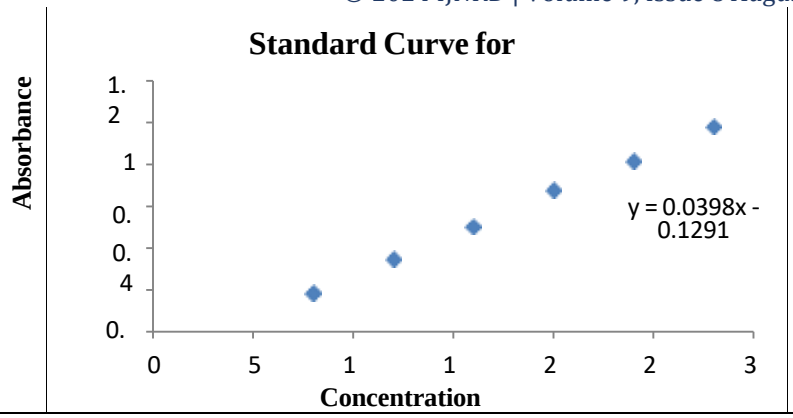


Figure 6: Standard Curve for Piracetam

8.1.4.1 Carbamazepine

The calibration curve of Carbamazepine was performed and graph plotted concentration vs. absorbance. The absorbance values of different concentration were noted. The regression equation was found to be y = 0.0398x-0.1291, with R^2 value of 0.9994. The graph was found to be linear.

Serial no.	Concentration (ppm)	Absorbance
1.	8	0.1868
2.	12	0.3484
3.	16	0.5037
4.	20	0.6791
5.	24	0.8167
6.	28	0.9838

Table 6: Concentration range and respective absorbance of Carbamazepine

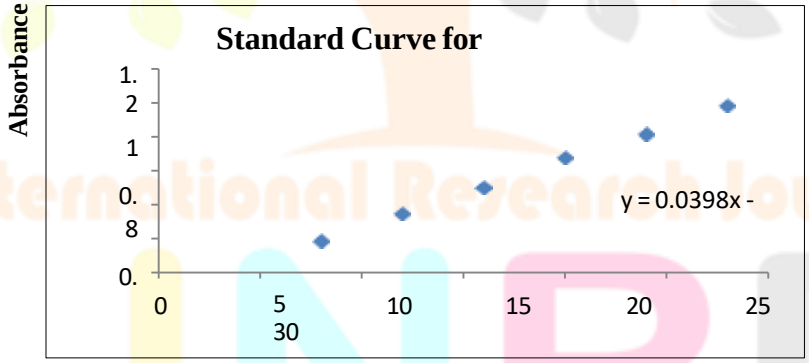


Figure 7: Standard Curve for Carbamazepine

8.2 Method Validation for Uv method development

8.2.1 Linearity

For the linearity of the Piracetam six point calibrations curve were plotted in a concentration range of 1-60 (µg/ml). From the linearity study it was observed that the drug was found to be linear in the concentration range and the linear regression equation was y = 0.0143x+0.0996 withcorrelation coefficient 0.9997. Whereas, for the Carbamazepine also six point calibrations curve were plotted in a concentration range of 8-28 (µg/ml). The equation was found to be y = 0.0398x-0.1291, with correlation coefficient of 0.9994

8.2.2 Accuracy

Accuracy of the proposed UV method for Piracetam and Carbamazepine was verified by conducting the recovery studies by using standard addition method. Standard drug concentration at three different percent levels was added to known amount of Piracetam and Carbamazepine. The percent recovery of added standards was calculated (Table 7).

The results showed better % mean recovery for respective percent levels. The % mean recovery values are closer to 100% showed high accuracy of the proposed UV analytical method.

Table 10: Evaluation data of Accuracy study of Acetazolamide

	Piracetam				
Concentration (%)	**Origin level (µg/ml)**	**Amount added (µg/ml)**	**% Recovery**	**Mean % Recovery**	**% RSD**
80	10	8	99.64	99.99	0.323
80	10	8	100.27		
80	10	8	100.08		
100	40	40	101.67	100.47	1.125
100	40	40	99.42		
100	40	40	100.34		
120	60	72	99.84	100.69	0.775
120	60	72	101.38		
120	60	72	100.85		
	Carbamazepine				
Concentration (%)	**Origin level (µg/ml)**	**Amount added (µg/ml)**	**% Recovery**	**Mean % Recovery**	**% RSD**
80	8	6.4	101.37	99.70	1.469
80	8	6.4	99.10		
80	8	6.4	98.63		
100	20	20	100.46	100.16	0.431
100	20	20	100.37		
100	20	20	99.67		
120	28	33.6	100.48	101.06	0.546
120	28	33.6	101.58		
120	28	33.6	101.12		

8.2.3 Precision

Intra-day and inter-day precision study of drug were evaluated for the 10 µg/ml, 40 µg/ml and60 µg/ml for Piracetam and 8 µg/ml, 20 µg/ml and 28 µg/ml for Carbamazepine.. Absorbance mean, percent assay and percent RSD were calculated for the intra-day as well as inter-day precision study (Table 8 and Table 9).

Table 8: Evaluation data for Intra-day and Inter-day study of Piracetam

Intra-day	**Morning**			**Afternoon**			**Evening**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
10	0.238	99.37	1.264	0.230	101.20	0.781	0.234	99.28	1.125
40	0.671	100.27	0.563	0.670	100.47	0.861	0.678	100.51	0.837
60	0.956	100.37	0.610	0.948	100.52	0.863	0.951	99.68	0.917
Inter-day	**Day 1**			**Day 2**			**Day 3**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
10	0.228	99.61	0.937	0.236	100.67	0.738	0.237	99.87	1.254

40	0.661	100.34	0.618	0.669	101.76	0.638	0.670	100.47	0.867
60	0.954	101.75	0.715	0.946	100.30	0.798	0.956	100.64	0.832

Table 9: Evaluation data for Intra-day and Inter-day study of Carbamazepine

Intra-day	**Morning**			**Afternoon**			**Evening**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
8	0.184	99.84	0.863	0.178	100.20	0.653	0.185	100.27	0.486
20	0.678	101.02	0.756	0.682	100.47	0.572	0.672	101.13	0.597
28	0.982	101.31	0.861	0.980	100.3	0.639	0.973	100.85	0.537
Inter-day	**Day 1**			**Day 2**			**Day 3**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
8	0.189	101.02	0.728	0.188	101.78	0.547	0.186	99.63	0.537
20	0.681	100.57	0.561	0.671	100.21	0.354	0.683	100.27	0.674
28	0.981	100.91	0.579	0.983	99.37	0.567	0.972	100.48	0.638

8.2.4 Robustness

Robustness study was evaluated by using three different solvent. The method was found to be robust as indicated by the % RSD values which are less than 2%. (Table 10)

Table 10: Evaluation data for Robustness of Piracetam & Carbamazepine

Piracetam			
Concentration (µg/ml)	**Solvents**	**Absorbance**	**% RSD**
40	Ethanol	0.674	0.480
40	Methanol	0.678	0.567
40	Distilled Water	0.681	0.631
Carbamazepine			
Concentration (µg/ml)	**Solvents**	**Absorbance**	**% RSD**
20	Ethanol	0.681	0.948
20	Methanol	0.690	0.864

8.2.5 Ruggedness

Ruggedness study of drug was carried out at the three different temperature levels. From the results it was found that the method was rugged showing the % RSD value less than 2%. (Table 11)

Table 11: Evaluation data for Ruggedness of Piracetam & Carbamazepine

Piracetam			
Concentration (µg/ml)	**Temperature (°C)**	**Absorbance**	**% RSD**
40	25	0.675	0.578
40	37	0.669	0.437
40	60	0.673	0.524
Carbamazepine			
Concentration (µg/ml)	**Temperature (°C)**	**Absorbance**	**% RSD**
20	25	0.683	0.506

20	37	0.689	0.571
20	60	0.672	0.463

8.2.6 Limit of Detection (LOD) & Limit of Quantification (LOQ)

Form the results it was found that LOD & LOQ are in the sub-microgram level, which indicates the sensitivity of the method. (Table 12)

Table 12: Evaluation data for LOD & LOQ of Piracetam & Carbamazepine

Piracetam	
LOD	0.457 µg/ml
LOQ	1.354 µg/ml
Carbamazepine	
LOD	0.211 µg/ml
LOQ	1.289 µg/ml

8.3 Method Development by Reverse Phase High Performance Liquid Chromatography

8.3.1 Optimization of Chromatographic Conditions and Method Development

In order to achieve the optimized chromatographic conditions to separate and quantify Piracetam and Carbamazepine one or two parameters were modified at each trial and chromatograms were recorded with all specified chromatographic Conditions. Various trials [figure 8-11] were carried out to finalize the optimized chromatographic conditions mentioned in the Table 13. Poorresolution, bad peak shapes, disturbances in base line were the few reasons of the rejections of the trials.

Table 13: Various Trials and Optimization of Chromatographic Conditions

Trial No	**HPLC System**	**Chromatographic Conditions**	**Observation**	**Remarks**
1	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase-**Methanol:Water80:20 **Column -** Inertsil C18 (4.6 x250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-** Isocratic **Column temperature-** Ambient **Wavelength-** 215nm	Peaks were not clearly separated. Base line isnot clear.	**Rejected**
2	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase-** Methanol and water (60:40) **Column -** Inertsil C18 (4.6 x250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-** Isocratic **Column temperature-** Ambient **Wavelength-** 215nm	Peaks were not clear. Base line is not clear.	**Rejected**
3	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase-**Methanol: Water50:50 **Column -** Inertsil C18 (4.6 x250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-** Isocratic **Column temperature-** Ambient **Wavelength-** 215 nm	Peaks shape were good, with good resolution and intensity	**Accepted**

Blank Chromatogram

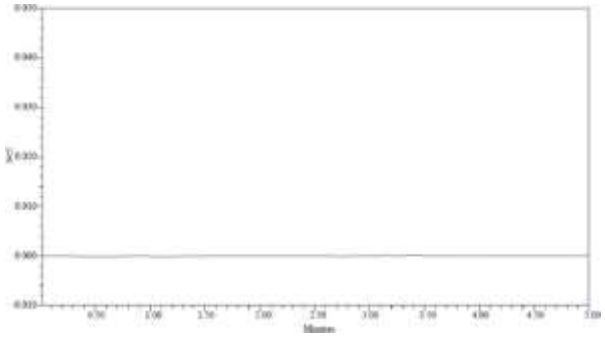
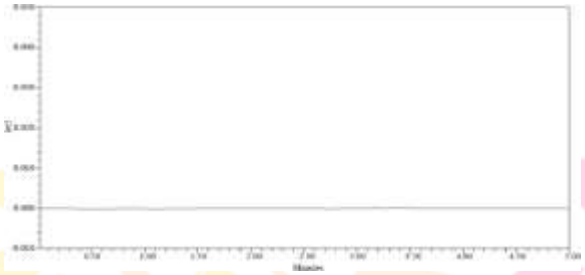


Figure 8: Blank Chromatogram

TRIAL 1



TRIAL 2

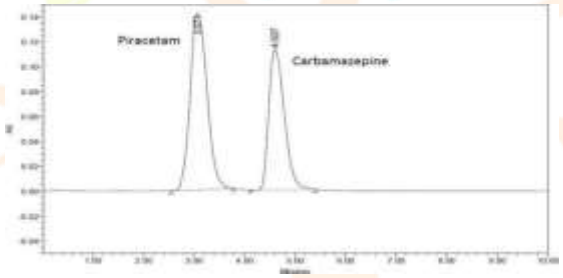


Table 15: Evaluation parameter of trial 2

Sr. No.	Name	Retention Time (min)	Area (µV*sec)	Height (µV)
1	Piracetam	3.056	3307638	140783
2	Carbamazepine	4.657	2397327	111635

TRIAL 3

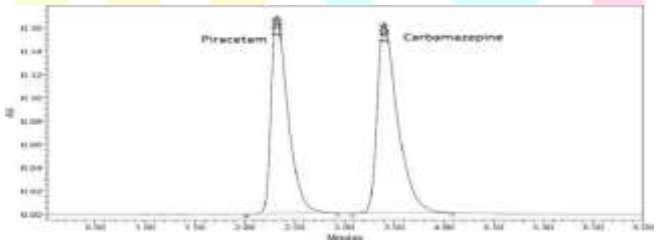


Figure 11: Optimized trial for HPLC Fingerprinting of Piracetam & Carbamazepine

Table 16: Evaluation parameter of optimized trial

Sr. No.	Name	Retention Time (min)	Area (µV*sec)	Height (µV)
1	Piracetam	2.326	3897263	165923
2	Carbamazepine	3.404	3196436	148846

8.4 Method Validation by RP-HPLC

The following parameters were considered for the analytical method validation of optimized method:

- System Suitability
- Specificity
- Linearity and Range
- Precision
  - ✓ System Precision
  - ✓ Method Precision
  - ✓ Inter-day Precision
  - ✓ Intraday Precision
- Ruggedness
- Accuracy (Recovery)
- Robustness
- Limit Of Detection(LOD)
- Limit Of Quantitation (LOQ)
- Solution Stability
- Application of method to the marketed dosage form

8.4.1 System Suitability

The HPLC method has been developed for the determination of the percentage assay of Piracetam and Carbamazepine in Tablet forms. The chromatograms of standard drugs alone and in their mixture are shown in figure 12, 13 and 14. The Retention time for Piracetam and Carbamazepine was found to be 2.32 & 3.40 min respectively and other parameters like, resolution, tailing factor, and theoretical plates were found to be within acceptable limit. (table 17)

Table 17: System Suitability Parameters for Piracetam & Carbamazepine

Sr. No.	Name	Retention Time*	Area*	USP Tailing*	USP Plate Count*
1	Piracetam	2.32	3897263	1.72	2252
2	Carbamazepine	3.40	3196436	1.73	2830

Figure 13: Standard Chromatogram of paracetam

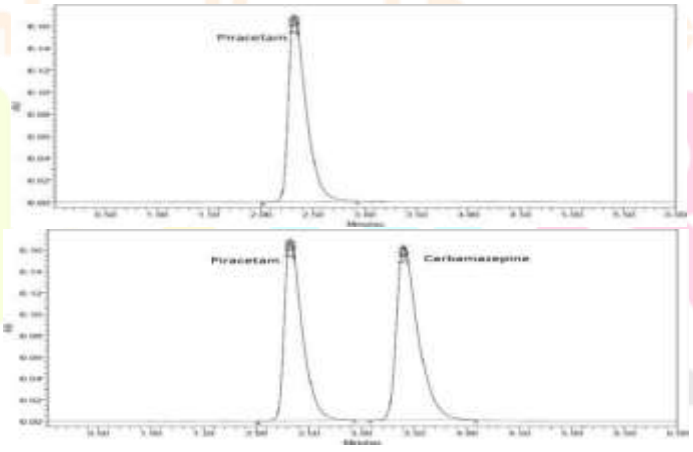


Figure 14: Standard Chromatogram of Mixture of Piracetam & Carbamazepine

8.4.2 Specificity

The absence of additional peaks in the chromatogram indicates non- interference of excipients. There was no interference from the blank at the retention time of analyte peaks. The peak purity data of sample solution was compared with standard solution. The peak purity plots are shown infigure 15-17 which reveals the homogenous peaks.

Figure 15: Blank Chromatogram

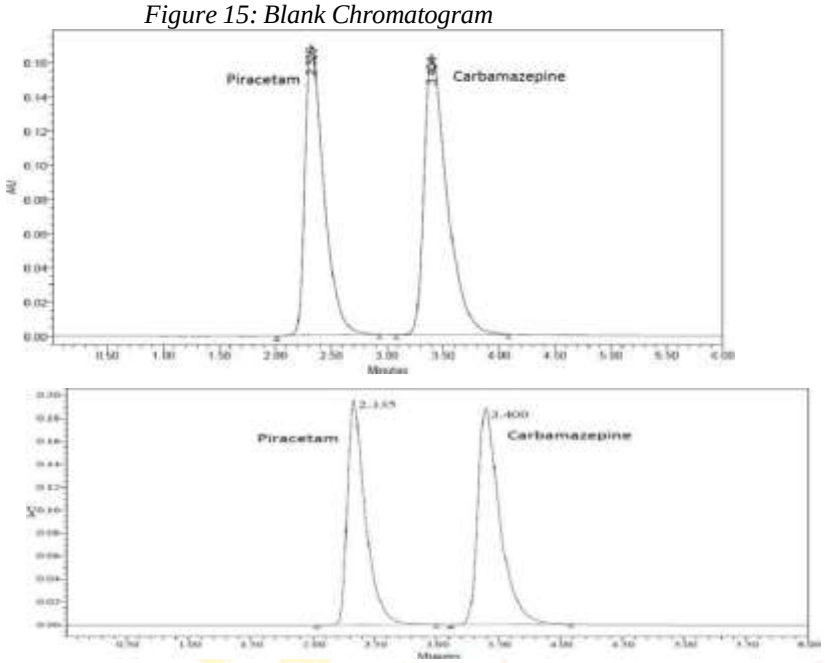


Figure 16: Purity Chromatogram of Standard Piracetam & Carbamazepine

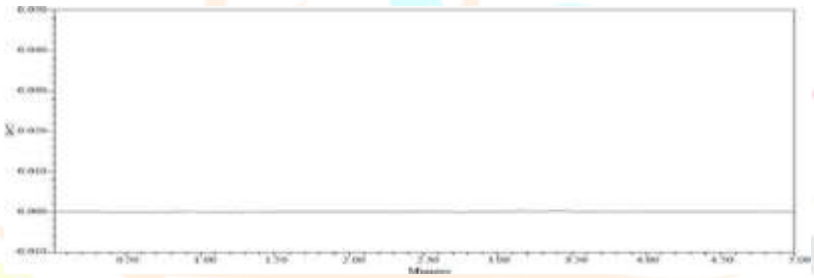


Figure 17: Purity Chromatogram of Sample of Piracetam & Carbamazepine

8.4.3 Precision

a) System Precision

The system precision was performed by measuring the peak response for standard drugs solutions in six replicates. Peak responses, mean, standard deviation and % relative standard deviation (%RSD) for Piracetam & Carbamazepine was found to be 0.160 and 0.027 %. The results are shown in table 18 and were found well within the acceptable criteria.

Table 18: System Precision Data of Piracetam & Carbamazepine

Sr. No.	Peak areas of Piracetam	Peak areas of Carbamazepine
1.	668978	784928
2.	1303018	1524159
3.	1984694	2329360
4.	2611374	3065982
5.	3269630	3830623
Mean	**1967539**	**2307010**
SD (±)	**1029333**	**1207013**
RSD (%)	**0.523**	**0.521**
Acceptance criteria	**% RSD should not be more than 2**	

a) Method Precision

The method precision was performed by measuring the peak response for sample solutions in sixreplicates. The % assay for Piracetam and Carbamazepine in six samples was calculated. The results of % assay and % RSD are shown in table 19. The chromatograms for the method precision are shown in fig ure18-23.

Table 19: Method Precision Data of Piracetam and Carbamazepine

Sample No.	% Assay of Piracetam(w/w)	% Assay of Carbamazepine(w/w)
1.	100.21	100.52
2.	99.26	101.34
3.	100.37	99.37
4.	100.24	100.28
5.	99.85	99.64
6.	99.46	100.54
Mean	**99.89**	**100.28**
SD (±)	**0.455**	**0.705**
RSD (%)	**0.455**	**0.705**
Acceptance criteria	**% RSD should not be more than 2**	

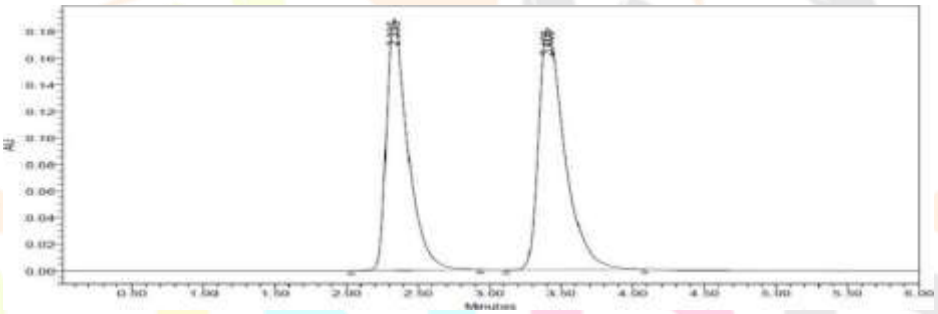


Figure 18: Chromatogram of Method precision 1

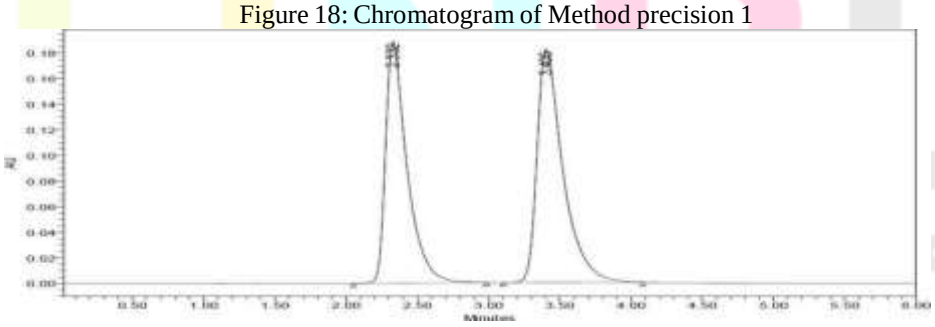
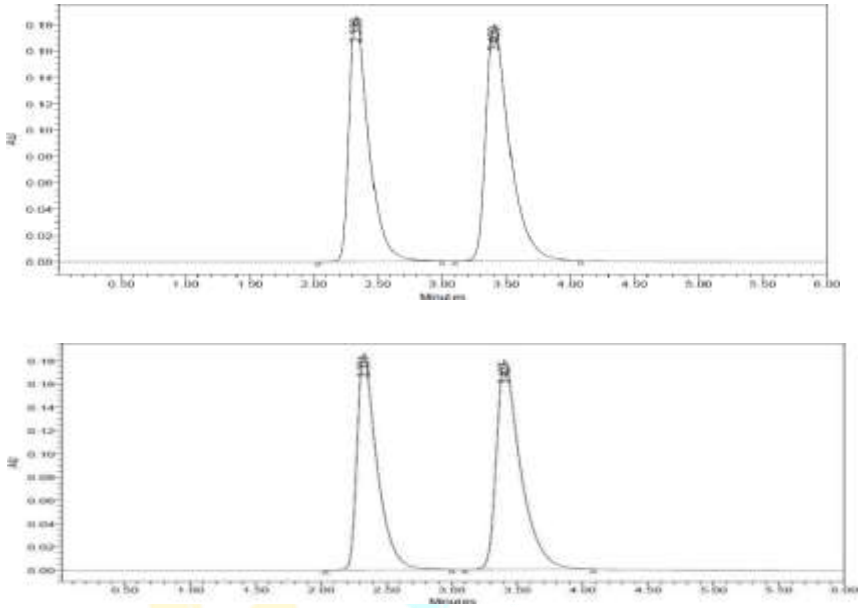


Figure 20: Chromatogram of Method precision 3

Figure 21: Chromatogram of Method precision 4



22: Chromatogram of Method pricision 5

Precision data of Piracetam and Carbamazepine

Piracetam	
Precision	**Area**
Precision-1	1963566
Precision -2	1964716
Precision -3	1965030
Precision -4	1960856
Precision -5	1966445
Precision -6	1964716
Mean	1964221
Standard Deviation	1889.07
%RSD	0.10
Carbamazepine	
Precision	**Area**
Precision-1	2304558
Precision -2	2299453
Precision -3	2296908
Precision -4	2295001
Precision -5	2299613
Precision -6	2299453
Mean	2299164
Standard Deviation	3221.30
%RSD	0.14

8.4.4 Accuracy (Recovery Study)

The accuracy of the assay method was evaluated by standard addition method in triplicate at 100 % level of the labeled claim and the percentage recovery was calculated. The mean % recovery was found to be 100.59 % & 100.46 % for Piracetam and Carbamazepine respectively. The results of the recovery study are shown in the table 23.

Table 23: Recovery study for Piracetam and Carbamazepine

Piracetam							
Level	**Set**	**Amount added(µg/ml)**	**Amount found(µg/ml)**	**%Recovery**	**Mean**	**SD**	**%RSD**
80%	10	8	7.89	99.57	100.26	0.654	0.652
	30	24	24.14	100.35			
	50	40	39.48	100.87			
100%	10	10	9.84	100.20	99.81	0.423	0.424
	30	30	29.64	99.36			
	50	50	50.34	99.87			
120%	10	12	11.28	100.37	100.10	1.031	1.030
	30	36	36.02	100.97			
	50	60	59.32	98.96			
Carbamazepine							
Level	**Set**	**Amount added(µg/ml)**	**Amount found(µg/ml)**	**%Recovery**	**Mean**	**SD**	**%RSD**
80%	8	6.4	6.34	99.67	99.74	0.464	0.465
	20	16	15.84	99.32			
	24	19.20	19.15	100.24			
100%	8	8	8.12	98.57	100.00	1.238	1.238
	20	20	21.05	100.74			
	24	24	23.42	100.69			
120%	8	9.6	9.65	100.84	100.13	0.737	0.736
	20	24	23.21	99.37			
	24	28.8	28.76	100.20			

8.4.5 Linearity and Range

Linearity for Piracetam and Carbamazepine was found to be in the range of 10 - 50µg /ml and 8 -24µg/ml respectively with correlation coefficient value (r2) 0.999 for both the drugs. The results were tabulated in table 24 and graphically represented in figure 24 and 30.

Table 24: Linearity and Range for Piracetam & Carbamazepine

Concentration in µg/ml for Piracetam	**Average Peak Area***	**Concentration in µg/ml for Carbamazepine**	**Average Peak Area***
10	668978	8	784928
20	1303018	12	1524159
30	1984694	16	2329360
40	2611374	20	3065982
50	3269630	24	3830623
Slope	**65097**	**Slope**	**763321**
CC	**14641**	**CC**	**17047**

Table 24: Linearity and Range for Piracetam & Carbamazepine

Concentration in µg/ml for Piracetam	Average Peak Area*	Concentration in µg/ml for Carbamazepine	Average Peak Area*
10	668978	8	784928
20	1303018	12	1524159
30	1984694	16	2329360
40	2611374	20	3065982
50	3269630	24	3830623
Slope	**65097**	**Slope**	**763321**
CC	**14641**	**CC**	**17047**

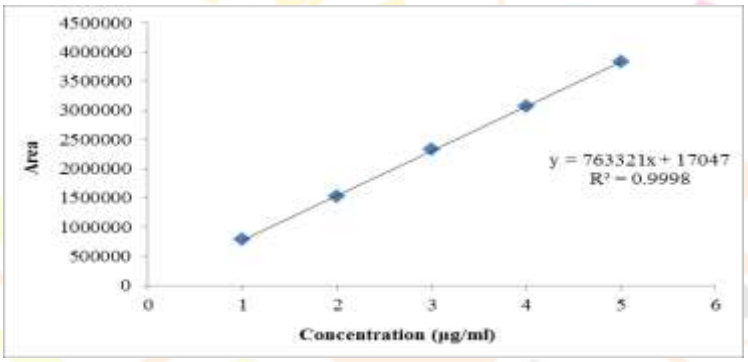


Figure 25: Standard Curve for Carbamazepine

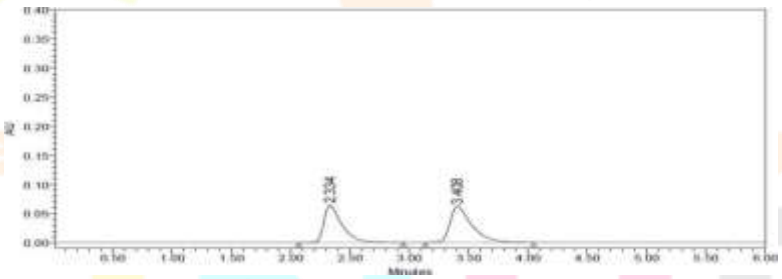
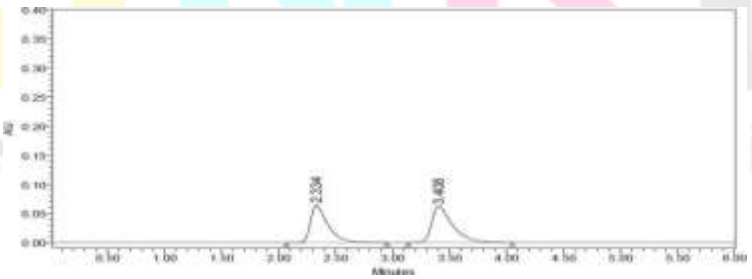


Figure26: Standard Chromatogram for Linearity 1



8.4.6 Stability in Analytical Solution

No significant difference was found in the % Assay of both drugs before and after storing for 24 hrs in refrigerator and room temperature. This confirms the stability of the drugs in solutions. The percentage assay is tabulated in table 25.

Table 25: Solution Stability Data of Piracetam & Carbamazepine

Time level	Refrigerator (25°C)	Room Condition (37°C)
Time in hrs	% Assay of Piracetam	% Assay of Carbamazepine
Initial	100.20 (±0.34)	99.38 (±0.032)
After 24 hrs	101.45 (±0.58)	100.02 (±0.046)

**Average of Six determination*

8.4.7 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

For Piracetam the LOD and LOQ were found to be 0.245µg/ml and 1.272µg/ml respectively. ForCarbamazepine LOD and LOQ were found to be 0.593µg/ml and 0.964µg/ml respectively. These values indicate that the method is suitable for the determination of the lower concentrationand confirms that proposed method is sensitive for the determination.

8.4.8 Robustness

The system suitability parameters and peak areas were evaluated in each condition and the results were compared with method precision results. %RSD at each condition was found less than 2. This indicates the robustness of the method. The results are tabulated in table 26.

Table 26: Robustness data of Piracetam & Carbamazepine

Flow rate	**Piracetam**				**Carbamazepine**			
0.8 ml/ min	**Rt**	**Area**	**Theoretical Plates**	**Tailing factor**	**Rt**	**Area**	**Theoretical Plates**	**Tailing factor**
Average	2.45	2292934	2934	1.621	3.58	2640647	2427	1.654
S. D	0.015	1221.02	156.84	0.0864	0.034	1235.5	152.67	0.0452
% RSD	**0.284**	**0.651**	**0.738**	**0.487**	**0.468**	**0.367**	**1.154**	**0.276**
1.2 ml/ min								
Average	2.34	2291656	2911	1.720	3.36	2639284	2336	1.768
S. D	0.038	1815.3	171.54	0.0634	0.014	1263.54	134.54	0.0137
% RSD	**0.438**	**0.687**	**1.286**	**0.574**	**0.495**	**0.768**	**0.547**	**0.867**

8.4.8 Robustness

The robustness parameter was determined by analyzing the different concentration at differenttemperature. The results were showed in table 27.

Table 27: Data of Robustness for Piracetam & Carbamazepine

Piracetam				
Change in Parameters	**Area of Standard**	**Mean**	**SD**	**%RSD**
25°C	1983627	1983928	911.96	**0.045**
	1984952			
	1983204			
37°C	1983275	1983524	217.37	**0.010**
	1983624			
	1983674			
60 °C	1984527	1983826	639.14	**0.032**
	1983674			
	1983276			
Carbamazepine				
Change in Parameters	**Area of Standard**	**Mean**	**SD**	**%RSD**
25°C	3067524	3066743	503.27	**0.016**
	3066248			
	3066726			
37°C	3067159	3067044	186.07	**0.006**
	3067143			
	3066829			
60 °C	3067426	3067190	303.79	**0.009**
	3066847			
	3067296			

9. CONCLUSION

The RP-HPLC method was developed and validated for the Simultaneous estimation of Piracetam & Carbamazepine in the bulk dosage form. The method was validated in accordance with ICH guidelines. All the validation parameters like linearity and range, accuracy, precision, specificity, system suitability, robustness and ruggedness have impact on the developed simultaneous method for the Piracetam and Carbamazepine. In addition, the main features of the developed method are short run time and retention time was observed on 2.32 min for Piracetam and 3.4 min for Carbamazepine.

In the current research, the method shows good reproducibility; moreover the RP-HPLC method is accurate, precise, specific, reproducible, sensitive and cost effective for the analysis of Piracetam & Carbamazepine.

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