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New Analytical Method Development and Validation and Stability Relatedd Impurity Studies of Rufinamide and Zonisamide Using RP-HPLC Method in Bulk and Pharmaceutical Dosage Form

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ABSTRACT

A simple, specific, accurate and stability indicating reversed phase high performance liquid chromatographic method was developed for the simultaneous determination of Rufinamide and Zonisamide in pure and tablet dosage form. A C-18 Develosil ODS HG-5 (150mm X 4.6mm i.d. 5µm) column in isocratic mode, with mobile phase containing Acetonitrile:Acetate buffer (60:40 v/v) adjusted to pH 4 using glacial acetic acid was used. The flow rate was 1.0 ml/min and effluents were monitored at 242 nm. The Retention time of Rufinamide and Zonisamide were 5.11 min and 7.63 min, respectively. The calibration curves were linear in the concentration range of 0-150 µg/ml for Rufinamide and 0-150 µg/ ml for Zonisamide. Rufinamide and Zonisamide stock solutions were subjected to acid and alkali hydrolysis, chemical oxidation and dry heat degradation. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time values. The proposed method was validated and successfully applied to the estimation of Rufinamide and Zonisamide in tablet dosage forms.

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INTRODUCTION

"Chromatography" is a method of separating a mixture of components into individual components through equilibrium distribution between two phases. High Performance Liquid Chromatography (HPLC) is one mode of chromatography, one of the most used analytical techniques. HPLC as compared with the classical LC technique is characterised by: High resolution. Small diameter (4.6 mm), stainless steel, glass or titanium columns.[1-5]

Column packing with very small (3, 5 and 10 μm) particles. Relatively high inlet pressures and controlled flow of the mobile phase. Continuous flow detectors capable of handling small flow rates and detecting very small amounts. Reverse phase chromatography is a bond phase chromatography technique, uses water as base solvent. Separation is based on solvent strength and selectivity. Separation is affected by column temperature and pH. In general, the more polar compounds elute faster than the less polar compounds. UV detection is the most common detection technique used.

According to ICH guidelines this following procedure is applicable to the development of new analytical methods by HPLC[3,4,6,7]. The simple meaning for method validation is a method which gives reliable results and checking the reliability of the results in all aspects. Other definitions include "Establishing documented evidence that a system does what it purports to do."[6-10]

FDA defines validation as "the documented program providing high degree of assurance that specific process or equipment will consistently produce product, meeting predetermined specification and quality attributes. USP has published specific guidelines for method validation for compound evaluation.

Forced degradation study by stressing active pharmaceutical ingredient (API) using Acid, Base, H_2O_2, Water and heat. If the molecule is known to be sensitive to light then stress the sample and sample solution with light.

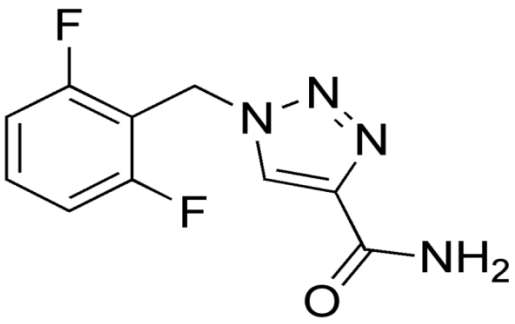
Subject the drug substance to stress with varied strengths of stressing agents to obtain degradation between 10% & 30%. Inject the samples into a HPLC system equipped with photo diode array (PDA) and check for separation of degradants formed under stressed conditions and the peak purity of the Active pharmaceutical ingredient (API) peak. [11-14]

If the purity of the peak is found to be satisfactory as per the individual software requirements, then the method can be considered as stability indicating. The API (Amlodipine & Rosuvastatin) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. This is one type of accelerated stability studies that helps us determining the fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing. The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.

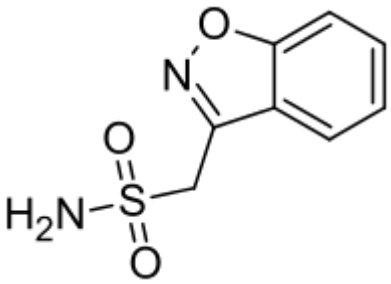
The main aim of the present investigation is, to undertake solubility and stability studies of Rufinamide and Zonisamide and to develop initial U.V. and chromatographic conditions. Setting up of initial UV and chromatographic conditions for the method development in pure and pharmaceutical dosage forms. Optimization of initial chromatographic and spectrophotometric conditions. Carry out assay of Rufinamide and Zonisamide with developed chromatographic conditions. Analytical method validation of the developed RP-HPLC method. Quantitative determination of Rufinamide and Zonisamide in pharmaceutical dosage form using the method developed and validated. To perform forced degradation studies of Rufinamide and Zonisamide with the developed method. The main objective is to develop a simple & accurate method for simultaneous estimation of rufinamide & zonisamide by using RP-HPLC.[15-18]

MATERIAL AND METHODS

Rufinamide:



Zonisamide:



Estimation of Maximum Wavelength by UV Spectroscopy: Accurately weighed quantities of the Rufinamide and Zonisamide (10mg each) were dissolved in methanol and the final volume was made up to 10ml, separately. The prepared solutions were scanned under UV region (200-400nm) for the estimation of maximum wavelength $\lambda_{max.}$

Preparation of solutions

Preparation of acetate buffer (pH-3-6)

Make up the following solutions

(1) 0.1M acetic acid

(2) 0.1M sodium acetate (tri-hydrate) (13.6g / l)

Mix in the following proportions to get the required pH

Table No. 1: Acetate buffer solutions pH 3 - 6

pH	vol. of 0.1M acetic acid	vol. of 0.1M sodium acetate
3	982.3 mls	17.7 mls
4	847.0 mls	153.0 mls
5	357.0 mls	643.0 mls
6	52.2 mls	947.8 mls

Preparation of Mobile Phase: The mobile phase used in this analysis consists of a mixture of Acetate Buffer (pH adjusted to 4.0 with glacial acetic acid) and Acetronitrile in a ratio of 60:40.

Preparation of Standard Stock Solutions and working standards: Accurately weighed around 25mg of Rufinamide & Zonisamide working standard, taken into a 25 ml volumetric flask, then dissolved and diluted to volume with the mobile phase to obtain a solution having a known concentration of about 1000 mcg/ml. Further dilutions has been made to get the final concentration of 100 µg/ml.

Preparation of Test solution: Diluted quantitatively an accurately measured volume of label claim solution with diluents to obtain a solution containing about a linear range.

Method development: Rufinamide & Zonisamide are relatively polar compounds. Preliminary attempts using reversed-phase HPLC using C_8 columns were not successful. Therefore, C_{18} Develosil ODS HG-5 RP 150mm x 4.6mm particle size5µm i.d. where analytes elute in order of decreasing polarity was selected for separation and quantification of drug. [19-21]

Selection of conditions:

Selection of Mobile phase: Mobile phase was selected based on solubility studies and on the literature survey.

Selection of organic Mobile phase: Acetonitrile was selected as the organic mobile phase as it provides good resolution for Rufinamide & Zonisamide.

Selection of Buffer and its pH: During the initial trials with phosphate buffer (pH 4.8) it was observed that the peak symmetry was not proper, hence to improve the resolution and peak shape, Acetate buffer was used. Then acetic acid was added to the buffer to adjust the pH 4 to improve the peak shape. The acetate buffer with varying pH (6.8, 5.2, 4.6 and 4.0) was tried. Symmetric peaks were observed best at pH 4.0.

Selection of Mobile phase composition: Varying proportions of acetate buffer (pH -4.6 adjusted with acetic acid) and acetonitrile were studied for the proper selection of ratio of the mobile phases.6:4 ACN:Buffer (% v/v) was found to be optimum since at this ratio no interference was observed with good resolution and peak purity.

Effect of flow rate: The flow rates of 0.5, 0.8 and 1.0 mL/min were used and chromatograms were recorded. At 1.0 mL/min symmetrical peaks with acceptable tailing factor were observed.

Effect of Column temperature: Separations were performed at three different column temperature 20^0C, 25^0C, 30^0C. However no specific change was observed upon changing the column temperature. For the present study 25^0C was selected.

Method validation:

Accuracy: For accuracy determination, three quality control samples were prepared i.e., 10 ppm, 25ppm and 50ppm of Rufinamide and Zonisamide injected in five replicate volumes of 20µL each. Accuracy is reported as the percent recovery of the known, added amount.

Acceptance criteria: The percentage recovery should be in the range of 85 to 115%.

Precision: Precision was determined by replicate processing. Precision was reported as Percent Relative Standard Deviation.10 ppm, 25ppm and 50ppm of Rufinamide and Zonisamide was selected to determine precision of the method. The Percentage Relative Standard Deviation for the areas were calculated (should not be more than 15%).

Acceptance criteria: The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

Linearity: Linearity of the developed method was demonstrated with Rufinamide and Zonisamide at six different concentrations from 1-100 ppm. Calibration QC standards were prepared fresh on the day of analysis by diluting the appropriate working solutions with mobile phase and injected into chromatographic system. The data were subjected to statistical analysis using a linear-regression model. The calibration curves were obtained by weighted linear regression (weighing factor $1/x^2$) using Microsoft Excel 2007 software. A graph was plotted with concentration versus peak area by covering six points.

Acceptance criteria: The plot for concentration versus peak area should be linear with a regression coefficient not less than 0.9990.

LOD and LOQ: LOD and LOQ was calculated according to ICH guidelines. The LOD and LOQ are shown in table 4-15. The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

$$L.O.D. = 3.3(SD/S).$$

$$L.O.Q. = 10(SD/S)$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

System suitability: System suitability was demonstrated using 50ppm Rufinamide and Zonisamide and 10µL volume of this solution was injected six times into the chromatographic system and the chromatogram was recorded. Results are shown in table 4-16.

System suitability was determined with the below mention parameters: Resolution, Capacity factor, Retention Time.

Stability related impurity studies:

Acid hydrolysis: An accurately weighed 25 mg. of pure drugs were transferred to a clean & dry 25 ml of two separate volumetric flask. To which 0.1 N Hydrochloric acid was added & make up to the mark & kept for 24 hrs. from both the volumetric flask 0.3 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of HCl (after all optimized conditions).

Basic hydrolysis: An accurately weighed 25 mg. of pure drugs were transferred to a clean & dry 25 ml of two separate volumetric flasks. To which 0.1 N Sodium hydroxide was added & make up to the mark & kept for 24 hrs. From both 0.3 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of NaOH (after all optimized conditions).

Thermal degradation: An accurately weighed 25 mg. of pure drugs were transferred to a clean & dry 25 ml of two separate volumetric flasks, make up to the mark with mobile phase. From this solution take 0.3 ml make up to the volume 10 ml & was maintained at 50 ^{0}C. for 24 hrs. then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

Photolytic Degradation: Approximately 10 mg. of pure drugs were taken in different clean & dry Petridish. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 0.3 mg. of each UV exposed drugs were transferred to a clean & dry 10 ml. volumetric flask. First the UV exposed drug was dissolved in mobile phase & make up to the mark then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

Oxidation with (3%) H_2O_2: Accurately weighed 10 mg. of pure drug was taken in a clean & dry 100 ml. volumetric flask. 30 ml. of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 ml. using water to prepare 100 ppm solution. The above sample was injected into the HPLC system. [22-37]

RESULTS AND DISCUSSION

Preliminary studies:

The solubility of Rufinamide and Zonisamide tested in Methanol, Ethanol Acetonitrile, hydrochloric acid, sodium hydroxide and Water.

Table No. 2: Solubility study of Rufinamide

Reagents	Solubility
Methanol	Sparingly soluble
Ethanol	Freely soluble
Acetonitrile	Soluble
1 (M) HCl	Sparingly Soluble
1(M) NaOH	Insoluble
Water	Insoluble

Table No. 3: Solubility study of Zonisamide

Reagents	**Solubility**
Methanol	Less soluble
Ethanol	Less soluble
Acetonitrile	Soluble
1 (M) HCl	Sparingly Soluble
1(M) NaOH	Insoluble
Water	Moderately soluble

Rufinamide was found to be sparingly soluble in HCL and methanol, soluble in acetonitrile. Zonisamide was found to be moderate soluble in methanol ethanol water and soluble in acetontrile.

UV-spectrophotometer analysis: The prepared solutions Rufinamide and Zonisamide (10mg each) were scanned under UV region (200-400nm) for the estimation of maximum wavelength $\lambda_{max.}$

Table No. 4: λ_{max} of Rufinamide & Zonisamide

S. No.	**Drug**	**λ_{max}**
1	Rufinamide	262nm
2	Zonisamide	228nm

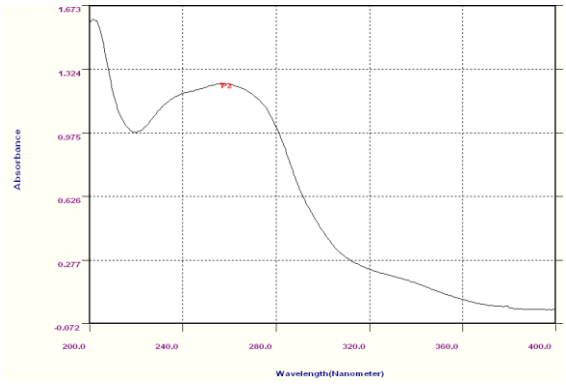


Fig. No. 1: UV-Spectrum for Rufinamide

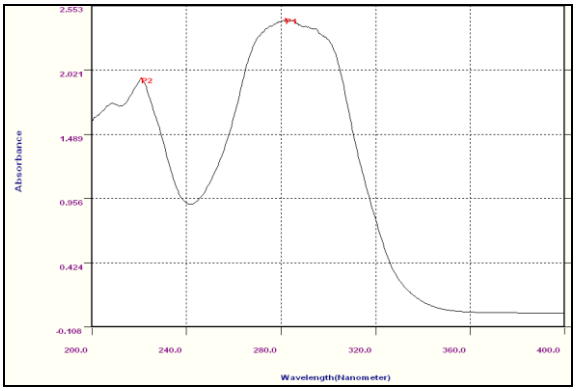


Fig. No. 2: UV-Spectrum for Zonisamide

From the absorption spectrum of Rufinamide & Zonisamide, λ_{max} was found to be at two wavelengths i.e., 262nm and 228nm. For the present study wavelength of 242nm was selected.

Method development:

Optimised condition: In intial Trails the resolution was very poor. To overcome this we changed the buffer, adjusted the pH and performed various trails by changing the mobile phase composition. In the ratio of 6:4 of ACN and Acetate buffer given the best results. The optimised conditions listed below.

Stationary phase	**:**	C_{18} Develosil ODS HG-5 (150mm x 4.6mm i.d, 5µm)
Mobile phase	**:**	Acetonitrile:Acetate buffer (6:4)
Elution mode	**:**	Isocratic
Sample concentration	**:**	100ppm.
Injection volume	**:**	20µL.
Run time	**:**	10 min.
Flow rate	**:**	1.0 ml/min.
Detection wavelength	**:**	242 nm.
Temperature	**:**	25^0C

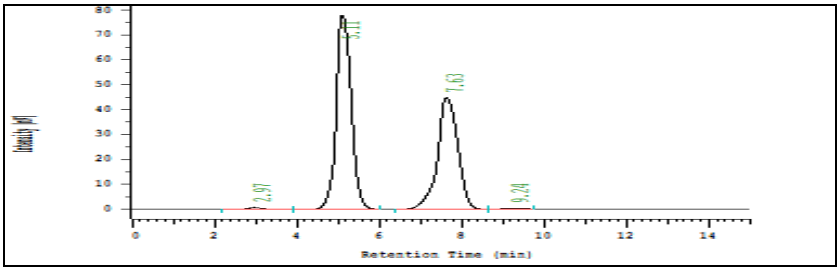


Fig. No. 3: Chromatogram for Optimised condition

Table No. 5: Peak integration data for optimised condition

Peak	Retention time (min)	Area	PEAK CONCENTRATION	Tailing factor
1	5.11	2037935	97.7	1.507
2	7.63	1473417	98.9	1.432

The peaks are well separated with good resolution and the tailing is minimised to acceptable range. Chromatogram is shown in figure 4-8 and peak integration data is shown in table 4-3.

Running the standard solution of Rufinamide

2 ml of stock solution prepared as mentioned under section 4.5.2 was pipetted out into a 10 ml volumetric flask. The volume was made up to the mark with methanol. The solution was filtered through the 0.45 µm membrane filter and degassed under ultrasonic bath prior to use. The solution was injected into the HPLC system.

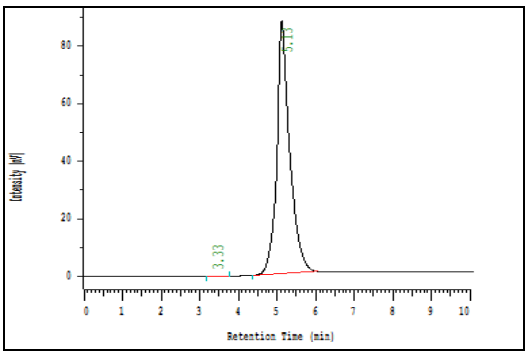


Fig. No. 4: Chromatogram of Rufinamide

Table No. 6: Peak integration data for Rufinamide

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.13	2037935	97.730

Retention time was found to be 5.13 min.

Running the standard solution of Zonisamide

5 ml of stock solution prepared as mentioned under section 4.5.3 was pipetted into a 10 ml volumetric flask. The volume was made up to the mark with methanol. The solution was filtered through the 0.45 μm membrane filter and degassed under ultrasonic bath prior to use. The solution was injected into the HPLC system.

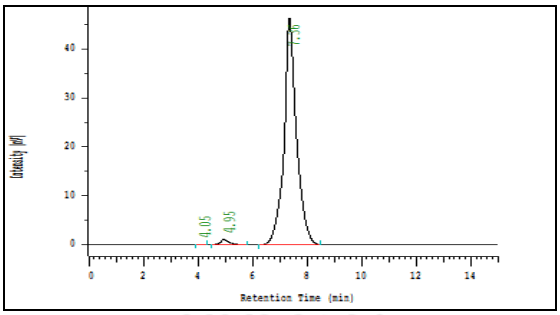


Fig. No. 5: Chromatogram of Zonisamide

Table No. 7: Peak integration data for Zonisamide

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	7.36	1473417	98.391

Retention time was found to be 7.36 min.

The HPLC system was set with the optimized chromatographic conditions to run the standard solution of Rufinamide & Zonisamide for 10 min. The retention time were found to be 5.13 min and 7.36 min respectively.

Validation results:

Accuracy and Recovery study:

Table No. 8: Accuracy data for Rufinamide

Accuracy Readings for 80% level

SAMPLE RUFINAMIDE (40+32PPM)	RT	PEAK AREAS	% RECOVERY
S_1 80%	5.17	1586487	96.87
S_2 80%	5.11	1598760	97.59
S_3 80%	5.13	1558933	95.24
AVG	5.136666667	1581393.333	96.56666667
S.D	0.030550505	20396.23893	1.204007198
%R.S.D	0.594753497	1.289763811	1.246814496

Accuracy Readings for 100% level

SAMPLE RUFINAMIDE(50+40PPM)	RT	PEAK AREAS	% RECOVERY
S_1 100%	5.11	1976221	95.87
S_2 100%	5.09	1967796	95.47
S_3 100%	5.07	1976002	95.86
AVG	5.09	1973339.667	95.73333333
S.D	0.02	4802.204737	0.228108161
%R.S.D	0.392927308	0.243354189	0.238274542

Accuracy Readings for 120% level

SAMPLE RUFINAMIDE(60+48PPM)	RT	PEAK AREAS	% RECOVERY
S_1 120%	5.07	2586059	103.85
S_2 120%	5.08	2582774	103.72
S_3 120%	5.07	2599284	104.36
AVG	5.073333333	2589372.333	103.9766667
S.D	0.005773503	0.338279963	0.338279963
%R.S.D	0.113800973	0.325342189	0.325342189

Average of % recoveries of Rufinamide

Concentration taken (µg/mL)	Recovery (%)
80	96.56
100	95.73
120	103.97

Table No. 9: Accuracy data for Zonisamide

Accuracy Readings for 80% level

SAMPLE ZONISAMIDE(40+32PPM)	RT	PEAK AREAS	% RECOVERY
S_1 80%	7.69	1271976	95.42
S_2 80%	7.61	1274668	95.62
S_3 80%	7.64	1277236	95.8
AVG	7.646667	1274626.667	95.61333333
S.D	0.0040415	2630.243588	0.190087699
% R.S.D	0.528525	0.206354037	198808777

Accuracy Readings for 100% level

SAMPLE ZONISAMIDE(50+40PPM)	RT	PEAK AREAS	% RECOVERY
S_1 100%	7.63	1598388	95.14
S_2 100%	7.59	1590827	94.7
S_3 100%	7.55	1594312	94.9
AVG	7.59	1594509	94.91333333
S.D	0.04	3784.347632	0.220302822
% R.S.D	0.527009	0.237336235	0.232109456

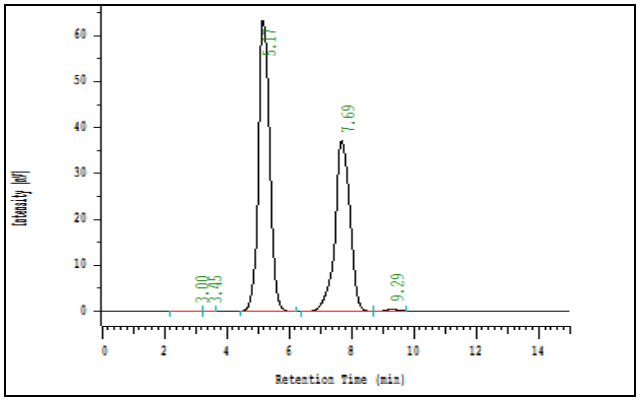
Accuracy Readings for 120% level

SAMPLE ZONISAMIDE(60+48PPM)	RT	PEAK AREAS	% RECOVERY
S_1 120%	7.55	2113768	104.02
S_2 120%	7.61	2122166	104.42
S_3 120%	7.3	2141158	105.8
AVG	7.586667	2125697.333	103.9766667
S.D	0.032146	14032.31133	104.7466667
% R.S.D	0.42371	0.660127437	0.891561347

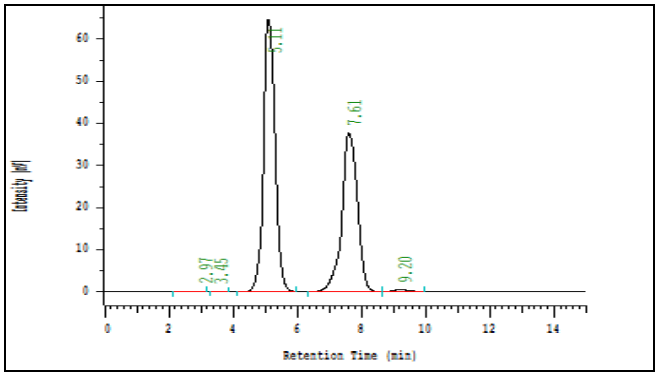
Average of % recoveries of Zonisamide

Concentration taken (µg/mL)	Recovery (%)
80	95.61
100	94.91
120	103.97

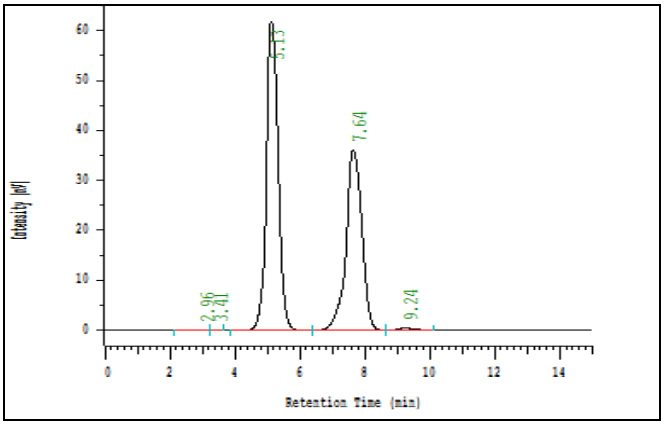
80% level



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.17	1586487	55.799
2	7.69	1241976	43.682



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.11	1598760	55.479
2	7.61	1374668	43.846



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.13	1558933	55.688
2	7.64	1227236	43.740

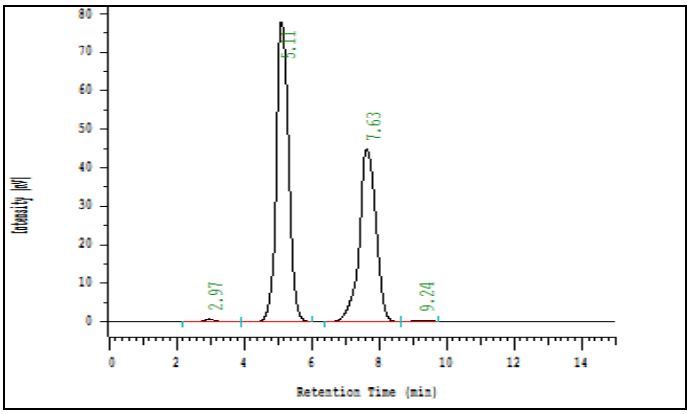
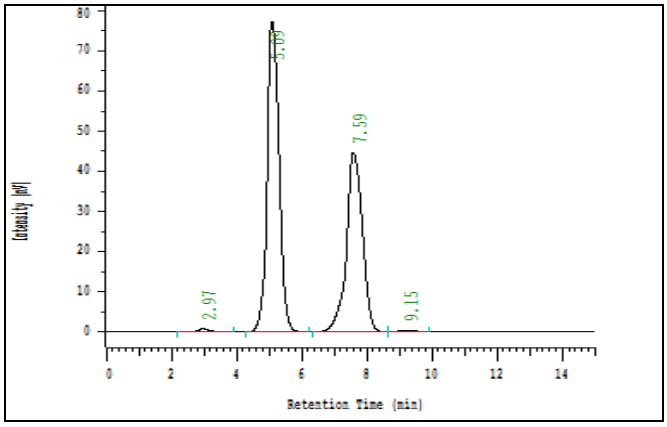
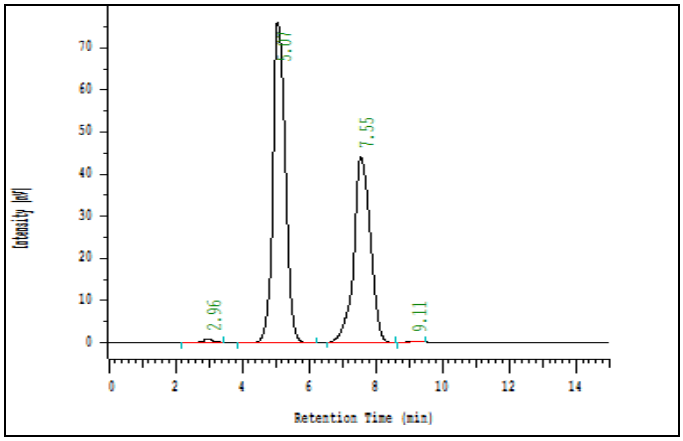


Fig. No. 6: Chromatograms and data for accuracy and recovery study 80% and 100% level

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.11	1976221	55.688
2	7.63	1598388	43.350



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.09	1967796	55.644
2	7.59	1590827	43.288



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.07	1976002	56.047
2	7.55	1594312	43.236

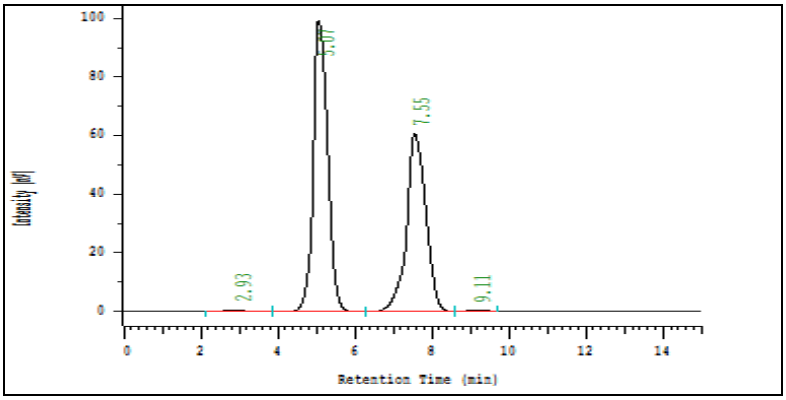
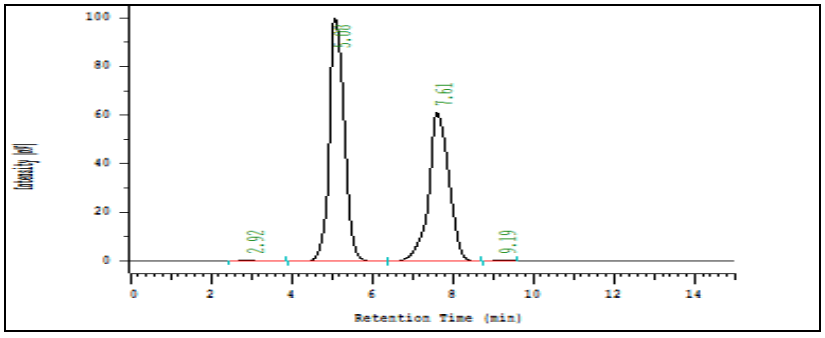


Fig. No. 7: Chromatograms and data for accuracy and recovery study 100% and 120% level

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.07	2586059	55.562
2	7.55	2113768	43.740



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.08	2582774	55.644
2	7.61	2122166	43.350

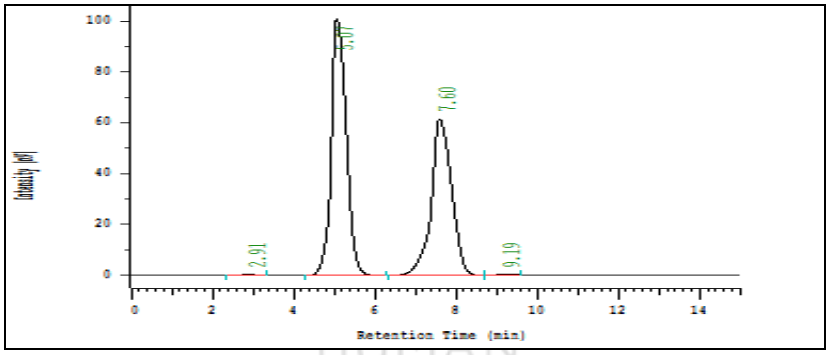


Fig. No. 8.: Chromatograms and data for accuracy and recovery study 120%

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.07	2599284	55.845
2	7.60	2141158	43.298

The mean recoveries were found to be 96.56, 96.73, 103.57 % for Rufinamide and 95.61, 94.91, 104.74% for Zonisamide The limit for mean % recovery is 95-105% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Specificity:

The interference of mobile phase, solvent and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of analytes is demonstrated in below chromatograms.

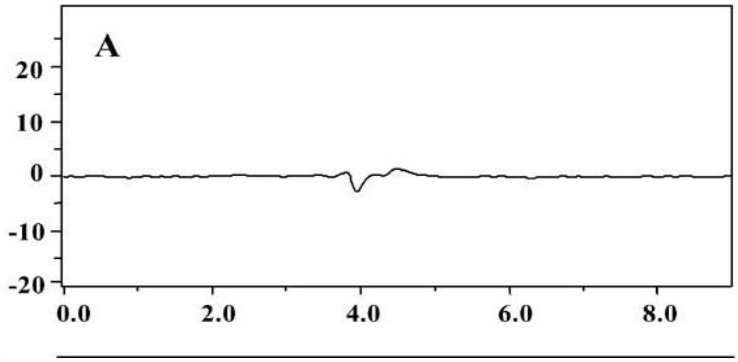


Fig. No. 9: Chromatogram for Blank in mobile phase

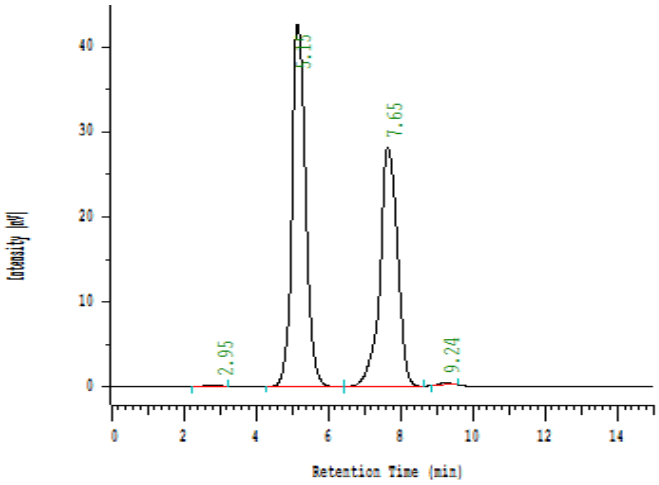


Fig. No. 10: Chromatogram for Rufinamide and Zonisamide

There are no interferences found in the analysis of the analytes. So the method is found to be specific for the given analytes.

Linearity:

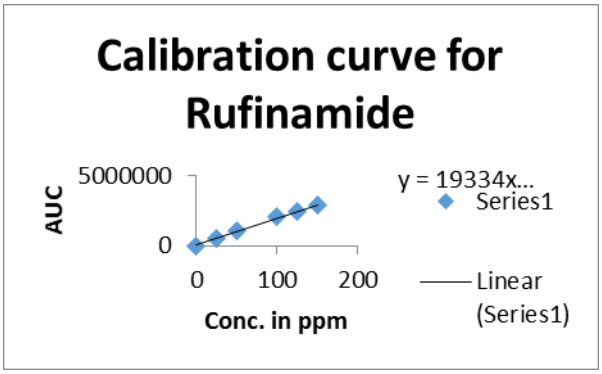


Fig. No. 11: Standard curve for Rufinamide

Table No. 10: Standard curve for Rufinamide

CONC.(µg/ml)	MEAN AUC
0	0
25	511367
50	1016983
100	2036487
125	2439569
150	2886059

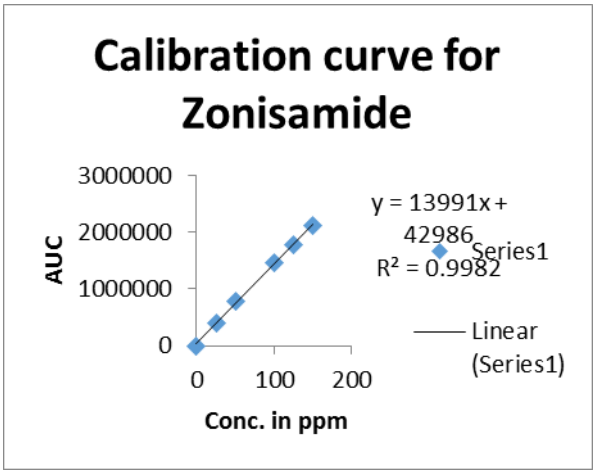
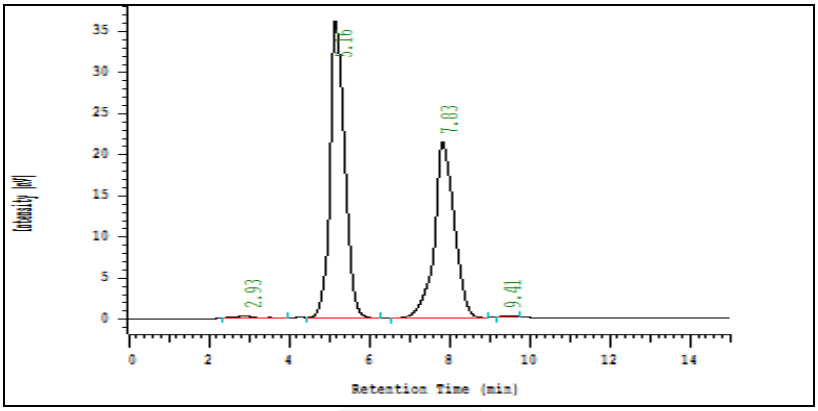


Fig. No. 12: Standard curve for zonisamide

Table No. 11: Standard curve for Zonisamide

CONC.	AUC
0	0
25	395359
50	791464
100	1471976
125	1781308
150	2113768



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.16	511367	54.985
2	7.83	395359	44.366

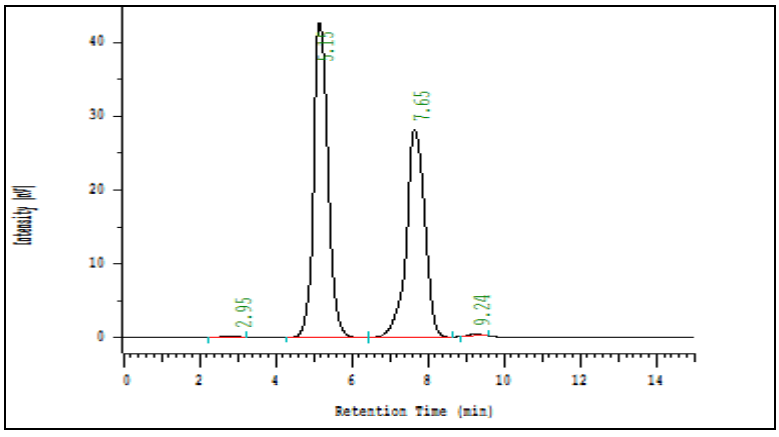


Fig. No. 13: Chromatograms and data of Linearity range for 25+25ppm

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.15	1016983	53.733
2	7.65	791464	45.771

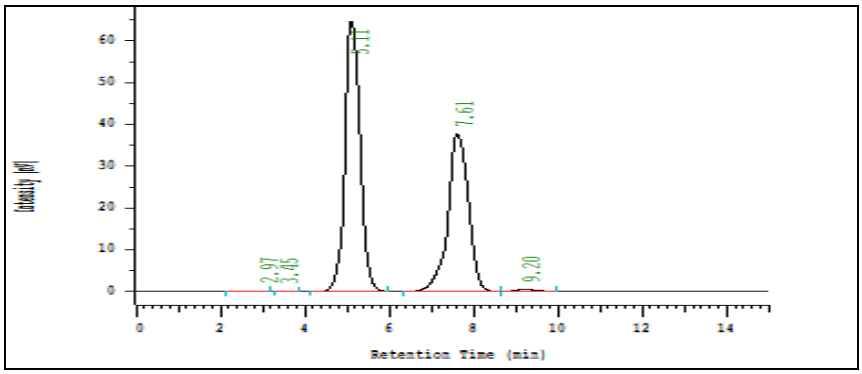


Fig. No. 14: Chromatograms and data of Linearity range for 50+50ppm

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.17	2036487	55.799
2	7.69	1471976	43.682

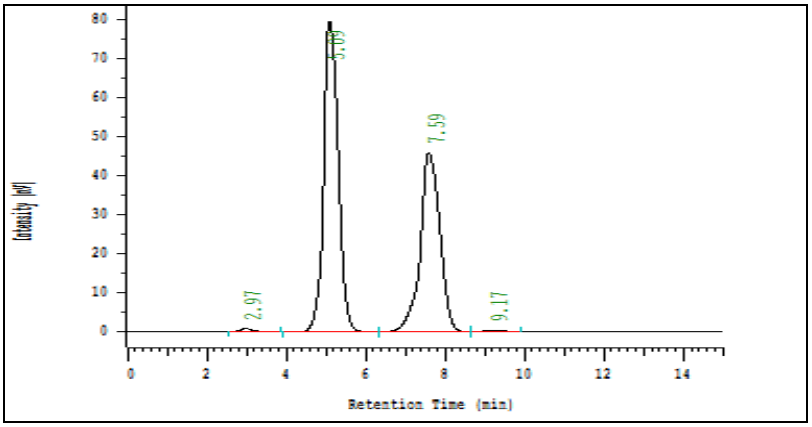


Fig. No. 15: Chromatograms and data of Linearity range for 100+100ppm

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.09	2439569	55.845
2	7.59	1781308	43.298

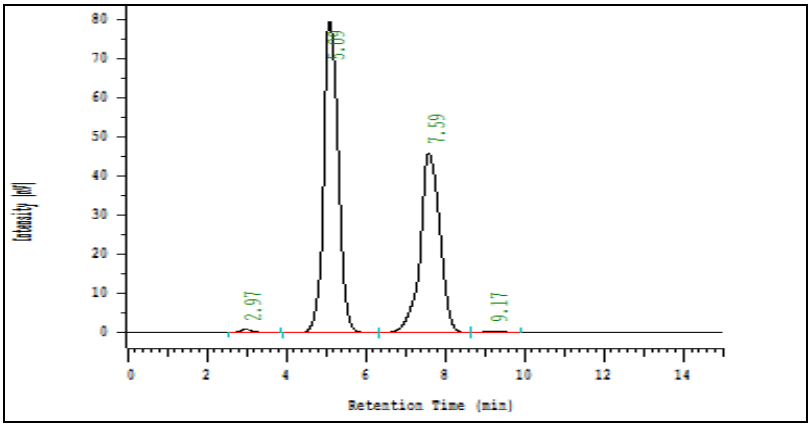


Fig. No. 16: Chromatograms and data of Linearity range for 125+125ppm

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.07	2886059	55.562
2	7.61	2113768	43.740

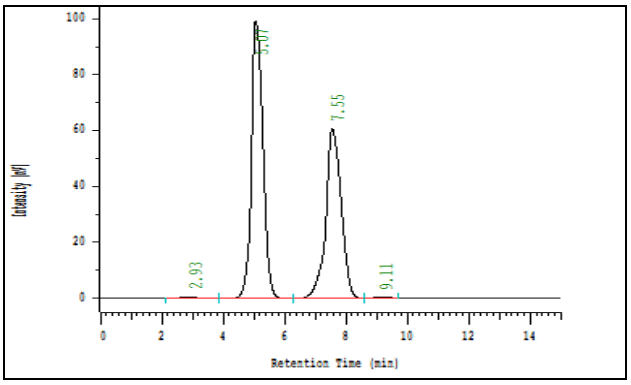


Fig. No. 17: Chromatograms and data of Linearity range for 150+150ppm

Linearity range was found to be 0-150 µg/ml for Rufinamide and 0-150 µg/ml for Zonisamide. The correlation coefficients were found to be 0.998 & 0.998, the slopes were found to be 19334 & 13991 and intercept were found to be 31664 & 42986 for Rufinamide & Zonisamide respectively.

Precision:

Repeatability

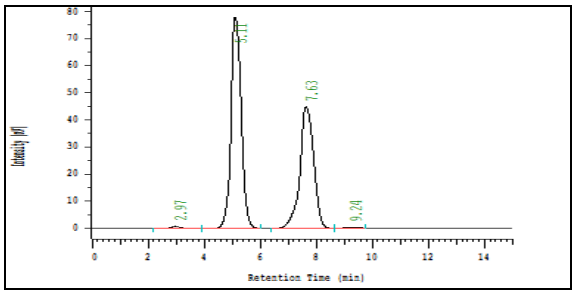
Table No. 12: **Data showing repeatability analysis for Rufinamide**

HPLC Injection Replicates of Rufinamide	**Retention Time**	**Area**
Replicate – 1	5.11	1976221
Replicate – 2	5.09	1967796
Replicate – 3	5.09	1999569
Replicate – 4	5.07	1976002
Replicate – 5	5.07	1963135
Average	5.086	1976544.6
Standard Deviation	0.016733201	14025.66844
% RSD	0.329005123	0.709605462

Table No. 13: **Data showing repeatability analysis for Zonisamide**

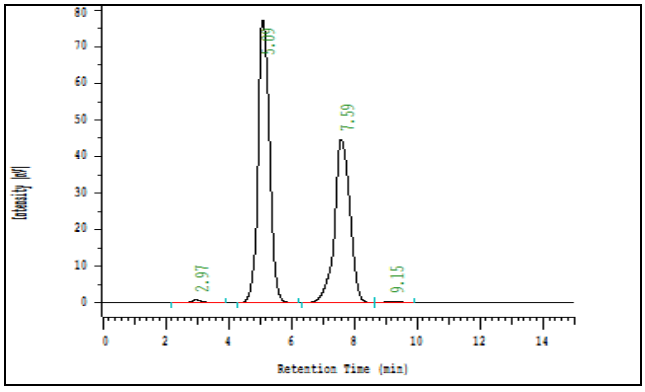
HPLC Injection Replicates of Zonisamide	**Retention Time**	**Area**
Replicate – 1	7.63	1538388
Replicate – 2	7.59	1530827
Replicate – 3	7.59	1558318
Replicate – 4	7.55	1524312
Replicate – 5	7.55	1540328
Average	7.582	1538434.6
Standard Deviation	0.033466	12808.78741
% RSD	0.441393	0.83258576

Repeatability 1



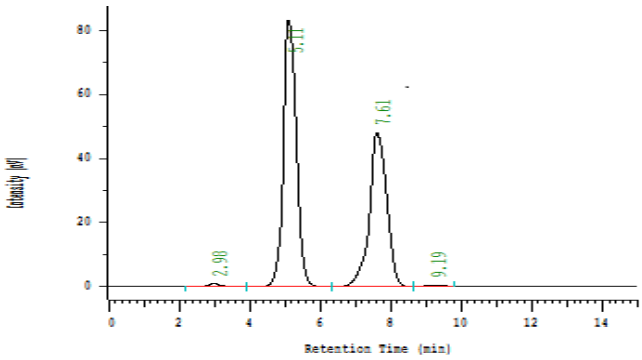
NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.11	1976221	55.688
2	7.63	1538388	43.350

Repeatability 2



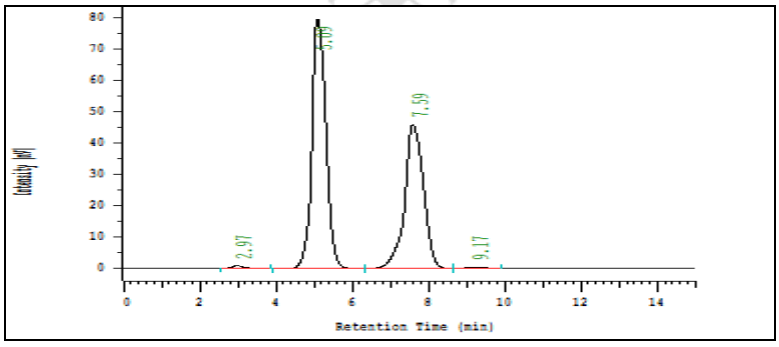
NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.09	1967796	55.644
2	7.59	1530827	43.288

Repeatability 3



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.11	1999569	55.845
2	7.61	1558318	43.298

Repeatability 4



NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.09	1976002	56.047
2	7.59	1524312	43.236

Repeatability 5

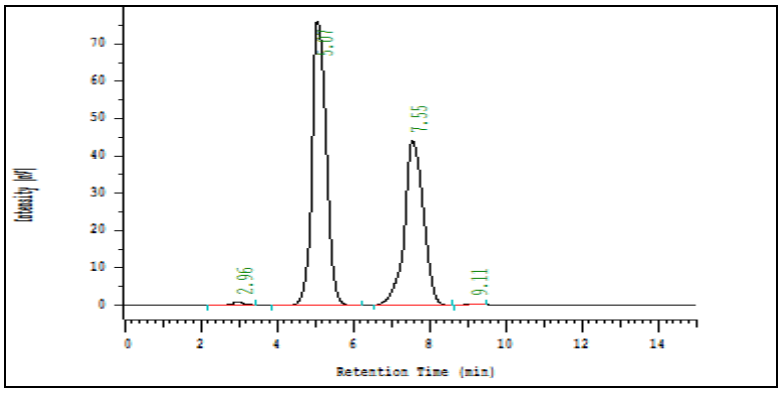


Fig. No. 18: Chromatograms and data of Repeatability

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.07	1963135	56.065
2	7.55	1540328	43.342

The repeatability study which was conducted on the solution having the concentration of about 40 µg/ml for Rufinamide and 50 µg/ml for Zonisamide (n =5) showed a RSD of 0.329005123% for Rufinamide and 0.441393% for Zonisamide. It was concluded that the analytical technique showed good repeatability.

Intermediate precision

Table No. 14: Data for Zonisamide analysis

Conc. Of Zonisamide (API) (µg/ml)	Observed Conc. of Zonisamide (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
50	50.02	0.86	50.03	0.87
100	99.14	0.30	100.03	0.32
150	149.57	0.13	149.95	0.11

Table No. 15: Data for Rufinamide analysis

Conc. Of Rufinamide (API) (µg/ml)	Observed Conc. Of Rufinamide (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
50	49.59	1.05	49.98	0.24
100	100.03	0.55	100.27	0.41
150	149.84	0.18	150.36	0.18

Intraday and interday studies show that the mean RSD (%) was found to be within acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within day and between days. Hence, method at selected wavelength was found to be precise.

LOD and LOQ:

The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

$$L.O.D. = 3.3(SD/S).$$

$$L.O.Q. = 10(SD/S)$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Table No. 16: Data of LOD and LOQ

S. no.	Parameter	Rufinamide	Zonisamide
1	LOD	0.32	1.44
2	LOQ	0.96	4.32

LOD (0.32+0.6 PPM)

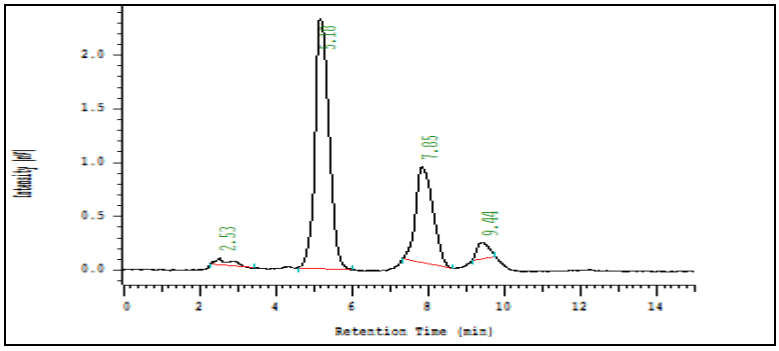


Fig. No. 19: Chromatogram and data of LOD

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.18	59472	64.423
2	7.85	28124	30.466

LOQ (0.96+4.32 PPM)

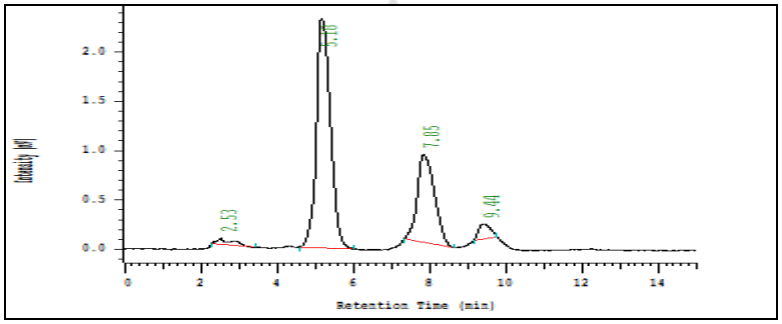


Fig. No. 20: Chromatogram and data of LOQ

NO	RT	PEAK AREA	PEAK CONCENTRATION
1	5.14	154133	53.024
2	7.81	107366	36.936

The LOD was found to be 0.32 µg/ml and 1.44 µg/ml and LOQ was found to be 0.96 µg/ml and 4.32 µg/ml for Rufinamide & Zonisamide respectively which represents that sensitivity of the method is high.

System suitability parameters:

Table No. 17: Data of System Suitability Parameter

S. No.	Parameter	Limit	Result
1	Resolution	Rs > 2	3.15
2	Asymmetry	T ≤ 2	Rufinamide =0.12 Zonisamide =0.5
3	Theoretical plate	N > 2000	Rufinamide =3246 Zonisamide= 4693

Method Robustness:

Table No. 18: Result of method Robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.07
Flow (0.9 ml/min)	0.02
Temperature (27^0C)	0.09
Temperature (23^0C)	0.13
Wavelength of Detection (244 nm)	0.04
Wavelength of detection (240 nm)	0.01

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^0C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-4, % RSD < 2%) the developed RP-HPLC method for the analysis of rufinamide & zonisamide(API).

Stability related impurity studies:

Rufinamide and Zonisamide stock solutions were subjected to acid and alkali hydrolysis, chemical oxidation and dry heat degradation. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time values.

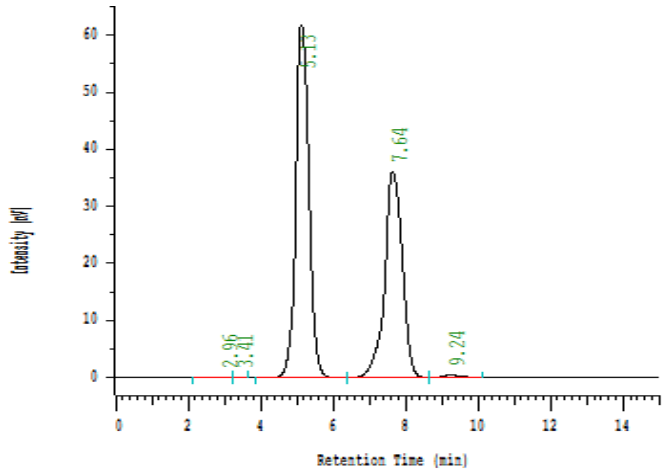


Fig. No. 21: Chromatogram showing degradation for Rufinamide & Zonisamide in 0.1 N HCl

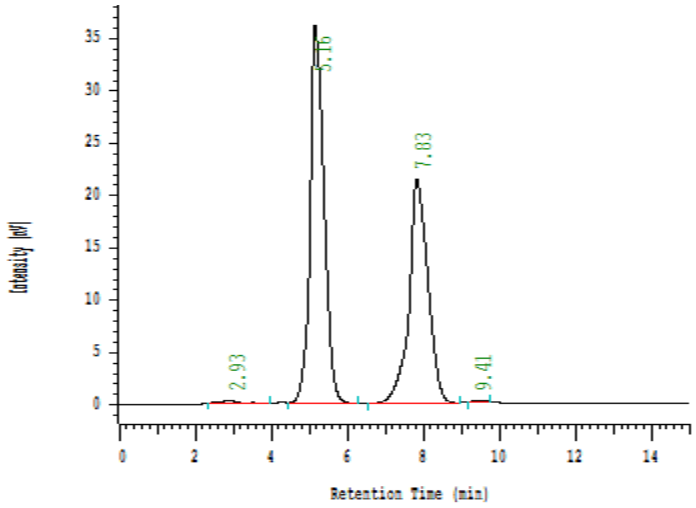


Fig. No. 22. Chromatogram showing degradation related impurity in 0.1 N NaOH

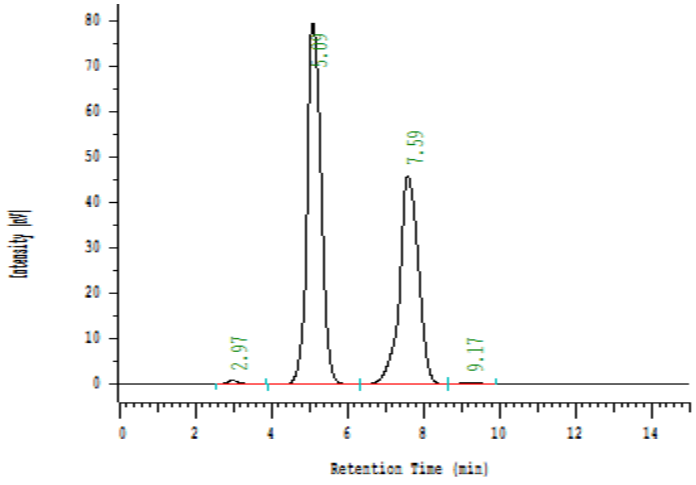


Fig. No. 23: Chromatogram showing thermal degradation studies

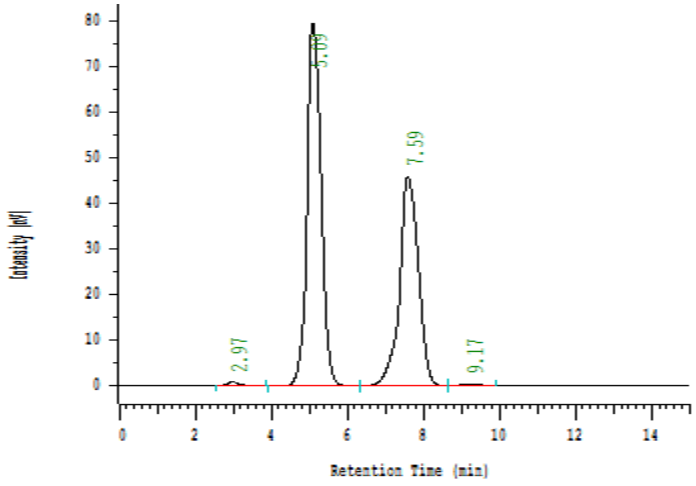


Fig. No. 24: Chromatogram showing photolytic degradation

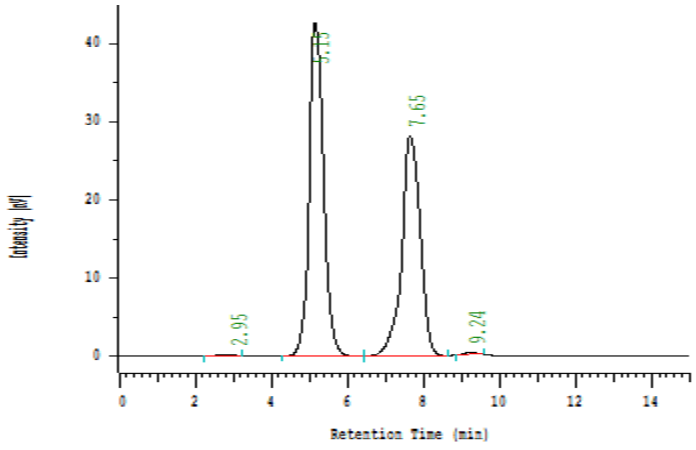


Fig. No. 25: Chromatogram showing oxidative degradation

Table No. 19: Results of stability studies of Rufinamide API

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	85.02	14.70	99.72
Basic Hydrolysis (0.I M NaOH)	24Hrs.	75.35	24.89	100.24
Thermal Degradation (50 ^{0}C)	24Hrs.	98.74	----------	98.74
UV (254nm)	24Hrs.	99.63	----------	99.63
3 % Hydrogen peroxide	24Hrs.	68.42	31.46	99.88

Table No. 20: Results of stability studies of Zonisamide API

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	62.48	35.78	98.26
Basic Hydrolysis (0.I M NaOH)	24Hrs.	84.26	15.19	99.45
Thermal Degradation (50 ^{0}C)	24Hrs.	98.85	----------	98.85
UV (254nm)	24Hrs.	98.74	----------	98.74
3 % Hydrogen peroxide	24Hrs.	82.95	17.28	100.23

The results of the stress studies indicated the specificity of the method that has been developed. Rufinamide & Zonisamide were almost stable in all stress conditions & areas reduced in acid, 3% H_2O_2 & basic stress conditions. We did not find any impurity peaks related to forced degradation or stability studies.

DISCUSSIONS:

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Rufinamide & Zonisamide, different chromatographic conditions were applied & the results observed are presented in previous chapters.

Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution.

In case of RP-HPLC various columns are available, but here develosil C_{18}, 5µm, 150 x 4.6 mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good.

Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, acetonitrile, dichloromethane, water, 0.1N NaOH, 0.1NHCl).

The drugs were found to be soluble in acetonitrile & sparingly soluble in methanol. Drug was insoluble in water. Using these solvents with appropriate composition newer methods can be developed and validated.

Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Rufinamide & Zonisamide it is evident that most of the HPLC work can be accomplished in the wavelength range of 200-250 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20 µl were found to be the best analysis.

The result shows the developed method is yet another suitable method for assay and purity which can help in the analysis of Rufinamide & Zonisamide in different formulations.

CONCLUSIONS:

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Rufinamide and Zonisamide API.

Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Rufinamide and Zonisamide dictated that the developed method is specific for the estimation of Rufinamide and Zonisamide.

Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

Even though no attempt has been made to identify the degraded products proposed method can be used as a stability indicating method for assay of Rufinamide and Zonisamide commercial formulations.

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