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RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF REBAMIPIDE

Nomula Srija* Rajitha S

KLR Pharmacy College, Paloncha, Bhadradri Kothagudem, Telangana- 507511, India.

Corresponding Author:- **Nomula Srija** Email:- **srijanomula007@gmail.com**

ABSTRACT

Rebamipide is widely used to treat gastric ulcers and mucosal damage, so its quantification in pharmaceutical formulations requires accurate and reliable methods. An RP-HPLC method for the estimation of Rebamipide in tablet dosage forms will be developed and validated in this study. Chromatographic separation was carried out on a C18 column using methanol and water as a mobile phase. The pH was adjusted to an optimal level and a suitable UV wavelength was employed for detection. Evaluations of specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness were conducted as part of the method validation process. The RP-HPLC method developed met all validation criteria and exhibited excellent performance over a specified concentration range and minimal variability, thus confirming its suitability for routine analysis of Rebamipide in pharmaceutical dosage forms.

Keywords: RP-HPLC, Method Development, Validation, Rebamipide.

INTRODUCTION

High-Performance Liquid Chromatography (HPLC) is a powerful analytical technique used for separating, identifying, and quantifying compounds in pharmaceutical, chemical, biological, and food industries (Arakawa, *et al*. 2010, 2. FDA. 2020). The high resolution, sensitivity, and reproducibility offered by HPLC make it a widely used method in quality control, formulation studies, and impurity profiling (ICH. 2005). HPLC allows the analysis of mixtures of closely related components that are difficult to separate by other conventional methods (Kazakevich Y & LoBrutto R. 2007).

Reverse Phase HPLC (RP-HPLC) is the most commonly used mode of HPLC. In RP-HPLC, the stationary phase is non-polar (hydrophobic) while the mobile phase is relatively polar. The retention time of analytes depends on their hydrophobic interactions with the stationary phase, with more non-polar molecules being retained longer (Liu X, *et al*. 2015). A typical RP-HPLC setup involves a C18 or C8 silica-based column, which provides good stability and separation efficiency (Moffat A, *et al*. 2011, Snyder L.R, *et al*. 2011)

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Basic Principle of RP-HPLC

In RP-HPLC, the separation occurs based on the partitioning of the analyte between the stationary and mobile phases. The non-polar stationary phase retains hydrophobic analytes more strongly, whereas more polar analytes pass through the column faster. As the mobile phase composition (such as water, acetonitrile, or methanol) is altered, analytes elute at different rates, producing a separation profile (retention time) for each component in the mixture (Wu L, *et al*. 2017).

RP-HPLC System Components

Pump:
Delivers the mobile phase at a constant flow rate through the system.

Injector:
Introduces the sample into the flow of the mobile phase.

Column:
The heart of the system where the separation occurs; RP columns are often packed with C18 silica.
Detector:
Identifies and quantifies the separated components; UV detectors are most common.

Data System:
Records chromatograms, calculates retention times, and quantifies analytes.

Stationary and Mobile Phases in RP-HPLC
Stationary Phase:
A hydrophobic material, usually silica coated with octadecylsilane (C18), making it non-polar.

Mobile Phase:
Composed of polar solvents such as water, methanol, or acetonitrile, often with pH modifiers like phosphate buffers.The mobile phase flows through the column, carrying analytes that partition based on their polarity and hydrophobicity (Kim Y, *et al.* 2018, Patel A.R, *et al.* 2020, Choudhury S, *et al.* 2018).

Applications of RP-HPLC in Pharmaceutical Analysis
RP-HPLC plays a crucial role in pharmaceutical analysis for:

- Quantitative estimation of active pharmaceutical ingredients (APIs) and excipients.
- Impurity profiling to ensure product safety.
- Dissolution testing in quality control laboratories.
- Stability testing of drugs under various environmental conditions.

Advantages of RP-HPLC

- Wide applicability for both polar and non-polar compounds.
- High resolution and reproducibility.
- Compatibility with a range of detectors (UV, fluorescence, MS).
- Automation capability for large sample throughput (Gupta V, *et al.* 2017, Deshmukh S. A & Patil V.R. 2019).

MATERIALS AND METHOD

Chemicals and Solvents
The reference sample of Rebamipide (API) was obtained from Macledos. The Formulation (Rebagen) was purchased from the local market. Methanol, Water used was of HPLC grade and purchased from Merck, Mumbai, India.

The mobile phase
A mixture of Methanol: water in the ratio of 90:10 v/v was prepared and used as mobile phase.

Standard solution of the drug
For analysis 100 ppm standard solution was prepared, required concentrations were obtained from 100 ppm solution by appropriate dilution.

Sample (tablet) solution
The formulation tablet of Rebagen (Rebamipide) was crushed to give finely powdered material. From the Powder prepared a 6 ppm solution in mobile phase and then filtered through Ultipor Ne6 Nylon 6, 6 membrane sample filter paper.

METHOD DEVELOPMENT

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate wave length and choice of stationary and mobile phases. The following studies were conducted for this purpose.

Trial 1:
Column: Waters C8 Column with 250 mmx4.6 mm i.d and 5 µm Particle size
Mobile Phase: MeOH: Potassium Di hydrogen ortho phosphate (0.02M) 50:50 v/v Buffer pH 7.0 adjusted with Ortho phosphoric acid
Flow rate: 1.0 ml/min
Wavelength: 279.0 nm
Temperature: Ambient
Injection Volume: 10 µl
Run time: 6 mins
Result: Chromatogram was shown in figure 4
Remarks: Peak shape was not good with slight disturbance at the end.

Trial 5:
Column: Waters C18 Column with 250 mmx4.6 mm i.d and 5 µm particle size
Mobile Phase: ACN: water: Potassium Di hydrogen ortho phosphate (0.02M) 30:30:40 v/v Buffer pH 6.0 adjusted with ortho phosphoric acid.
Flow rate: 1.0 ml/min
Wavelength: 279.0 nm
Temperature: Ambient
Injection Volume: 10 µl
Run time: 12 mins
Result: Chromatogram was shown in figure 5
Remarks: Peak shape was not good and tailing factor was more.

Detection wavelength
The spectrum of l0 ppm solution of the Rebamipide in methanol was recorded separately on UV spectrophotometer. The peak of maximum absorbance

wavelength was observed. The spectra of Rebamipide were showed maximum absorbance at 210nm.

Choice of stationary phase

Preliminary development trials have performed with octadecyl columns with different types, configurations and from different manufacturers. Finally the expected separation and peak shapes were obtained on chromosil Cl8 (250 mm x 4.6 mm, 5pm) column.

Selection of the mobile phase

In order to get sharp peak, low tailing factor and base line separation of the separation of the components, a number of experiments were carried out by varying the composition of various solvents and flow rate. To have an ideal separation of the drug under isocratic conditions, mixtures of solvents like methanol, water and Acetonitrile with or without different buffers indifferent combinations were tested as mobile phases on a Chromosil Cl8 column. A mixture of Methanol: water in the ratio of 90:10 v/v was proved to be the most suitable of all the combinations since the chromatographic peak obtained was better defined and resolved and almost free from tailing.

Flow rate

Flow rates of the mobile phase were changed from 0.5 - 1.5 mL/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

Optimized chromatographic conditions

Chromatographic conditions as optimized above were shown in Table 6.1. These optimized conditions were followed for the determination of Rebamipide in bulk samples and in its Formulations. The chromatogram of standard (4ppm) shown in Figure 6.

VALIDATION OF THE PROPOSED METHOD

The proposed method was validated as per ICH guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, robustness, system suitability, limit of detection, limit of quantification, and solution stability.

Specificity

The specificity of method was performed by comparing the chromatograms of blank, standard and sample (Prepared from Formulation). It was found that there is no interference due to excipients in the tablet formulation and also found good correlation between the retention times of standard and sample. The specificity results are shown in Table 1.

Linearity

Linearity was performed by preparing mixed standard solutions of Rebamipide at different concentration levels including working concentration mentioned in experimental condition i.e. 4 ppm. Twenty micro liters of each concentration was injected in duplicate into the HPLC system. The response was read at 210 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The regressions of the plots were computed by least square regression method. Linearity results were presented.

Precision

Precision is the degree of repeatability of an analytical method under normal Operational conditions. Precision of the method was performed as intraday precision, Inter day precision.

Intraday precision

To study the intraday precision, six replicate standard solutions (2ppm) of Rebamipide were injected. The percent relative standard deviation (% RSD) was calculated and it was found to be 0.465, which are well within the acceptable criteria of not more than 2.0.

Inter Day precision

To study the interday precision, six replicate standard solution of Rebamipide was injected on third day of sample preparation. The percent relative standard deviation (% RSD) was calculated and it was found to be 1.025, which are well within the acceptable criteria of not more than 2.0.

Accuracy

The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at 50%, 100% and 150% level of 20ppm. The solutions were analyzed in triplicate at each level as per the proposed method. The percent recovery and % RSD was calculated and results are presented in Table 2 Satisfactory recoveries ranging from 99.0 to 102.0 were obtained by the proposed method. This indicates that the proposed method was accurate.

Robustness

The robustness study was performed by slight modification in flow rate of Mobile phase, pH of the buffer and composition of the mobile phase. Rebamipide at 2 ppm concentration was analyzed under these changed experimental conditions. It was observed that there were no marked changes in chromatograms, which

demonstrated that the developed method was robust in nature.

System suitability

System suitability was studied under each validation parameters by injecting six replicates of the standard solution 2 ppm). The results obtained were within acceptable limits (Tailing factor < 2 and Theoretical plate's > 2000) and are represented in Table 3. Thus, the system meets suitable criteria.

Limit of detection and Limit of quantification

Limit of detection (LOD) is defined as the lowest concentration of analyte that gives a detectable response. Limit of quantification (LOQ) is defined as the lowest Concentration that can be quantified reliably with a specified level of accuracy and Precision. For this sample was dissolved by using Mobile Phase and injected until peak was diapered. After 0.05ppm dilution, Peak was not clearly observed. So, it confirms that 0.05 ppm is limit of Detection. For this study six replicates of the analyte at lowest concentration were Measured and quantified. The LOD and LOQ of Rebamipide are given in Table 4.

Table 1: Specificity study.

Name of the solution	Retention Time in Min
Blank	**NO PEAKS**
Rebamipide	**3.8**

Table 2: Percentage Recovery and Percentage RSD.

Level	Amount of Rebamipide spike (PPM)	Amount of Rebamipide Recovered (ppm)	% Recovery	%RSD
50%	3 ppm	2.95	99.33	0.191
	3 ppm	2.97	99.0	
	3 ppm	2.96	99.34	
100%	4 ppm	3.98	99.5	0.383
	4 ppm	3.94	99.0	
	4 ppm	3.99	99.75	
150%	6 ppm	5.99	99.81	0.170
	6 ppm	6.0	100.0	
	6 ppm	5.92	99.66	
			Mean % of recovery 99.48	**Mean RSD 0.248**

Table 3: Results of System suitability

Parameter	Tailing factor	Theoretical plates
Specificity study	1.18	4034
Linearity study	1.60	3646
Precision study	1.36	5378

Table 4: LOQ and LOD.

Parameter	Measured Volume
Limit of Quantification	0.165 ppm
Limit of Detection	0.05 ppm

Figure 1: Trial 1 Chromatogram of Rebamipide.

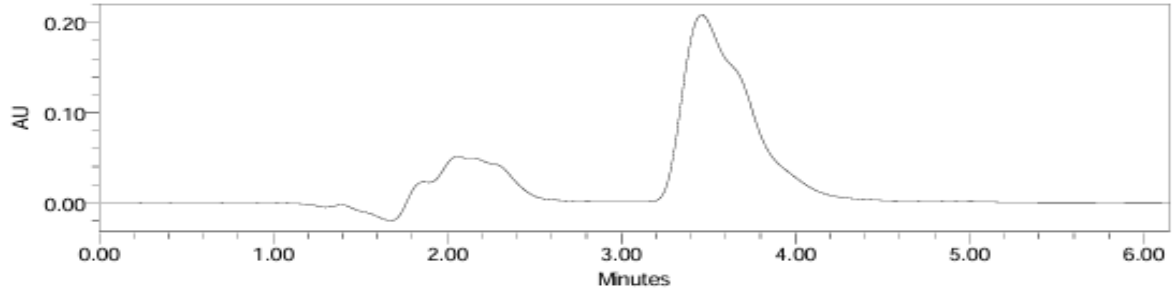


Figure 2: Trial 2 Chromatogram of Rebamipide.

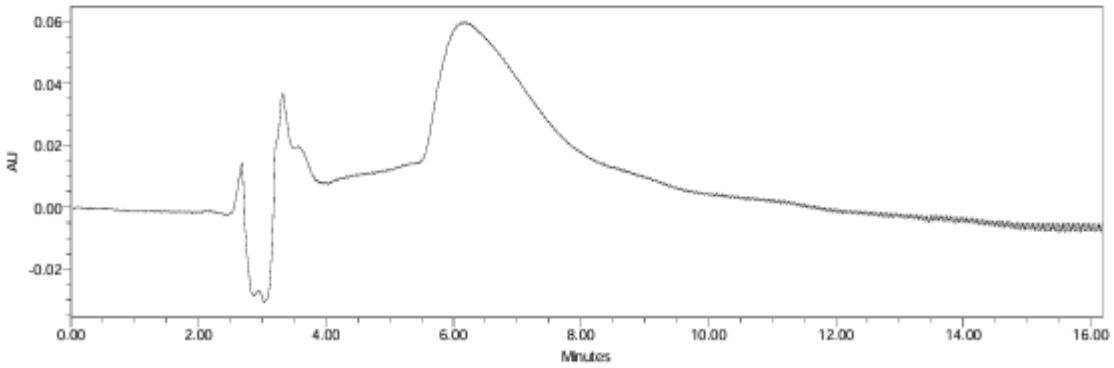


Figure 3: Trial 3 Chromatogram of Rebamipide.

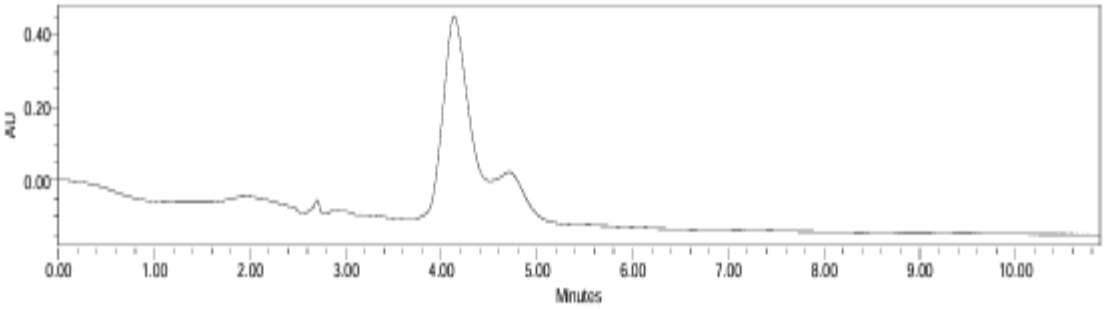


Figure 4: Trial 4 Chromatogram of Rebamipide.

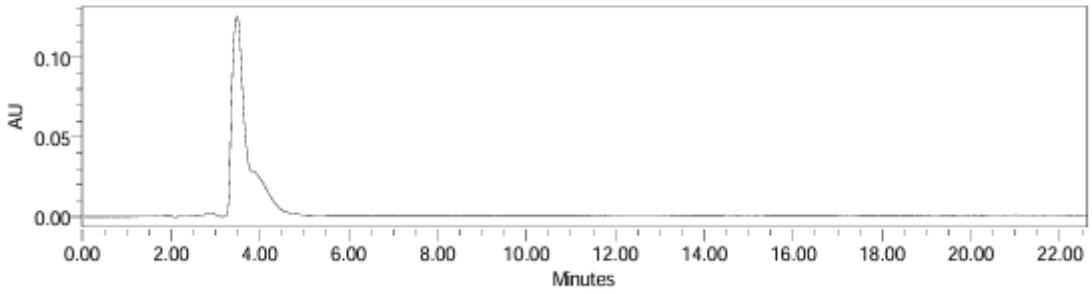


Figure 5: Trial 5 Chromatogram of Rebamipide.

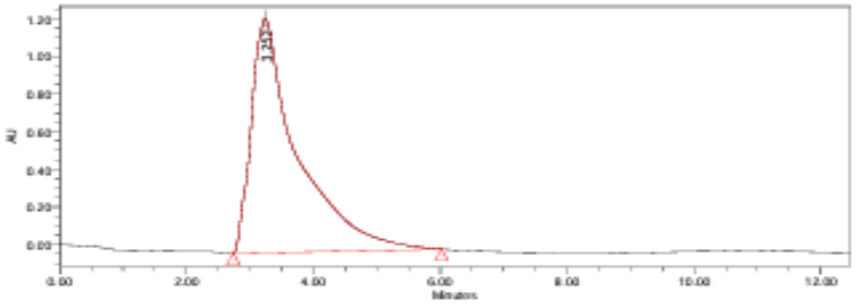


Figure 6: Chromatogram of standard solution

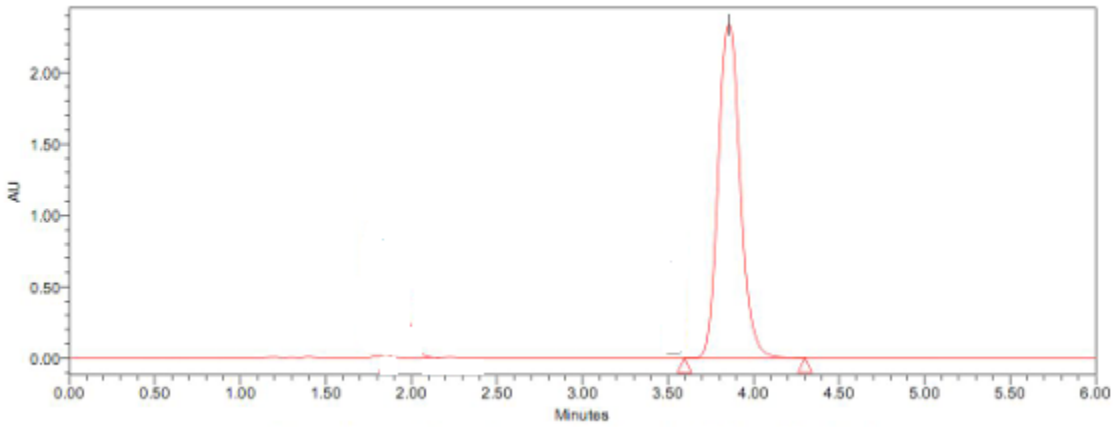
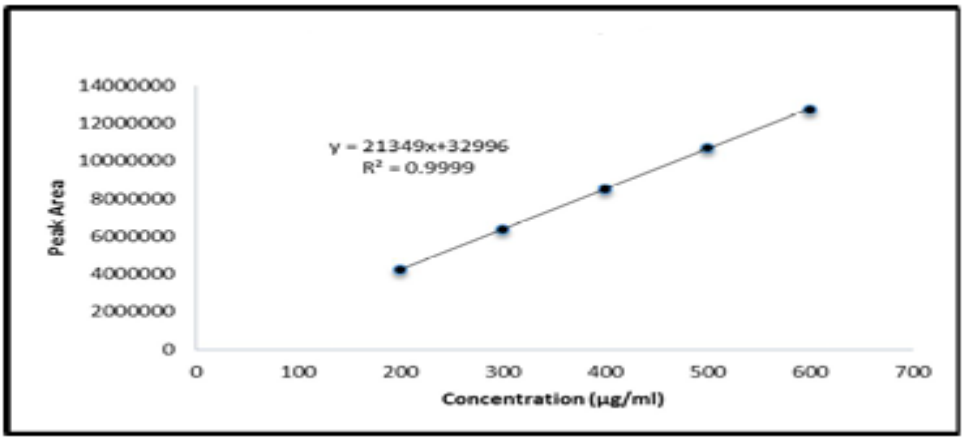


Figure 7: Linearity graph.



RESULTS AND DISCUSSION

To develop a precise, accurate and suitable RP-HPLC method for the simultaneous estimation of Rebamipid**e** different mobile phases were tried and the proposed chromatographic conditions were found to be appropriate for the quantitative determination. Proper selection of the stationary phase depends up on the nature of the sample, and molecule Physico- chemical properties. Mixture of Methanol and Water (90:10 v/v) was selected as mobile phase and the effect of composition of mobile phase on the retention time of Rebamipide was thoroughly investigated. The concentration of the Water and methanol were optimized to give symmetric peak with short run time. A system suitability test was applied to representative chromatograms for various parameters. The results obtained were within acceptable limits and are represented in Table 6.3. Thus, the system meets suitable criteria. Six points linear curve was constructed covering a concentration range of 1- 6ppm (Three independent determinations were performed at each concentration). A linear relationship was observed between peak response and the concentration of Rebamipide. The linear regression equation was y = -1299 + 89210x (i= 0.999). The R.S.D. values of the slope were 79294.52 (n=3) and the R.S.D. of y-intercept was 2117.43 (n=3). Linearity values are shown in Table 5.4. The data of regressing analysis of the calibration curves are shown in Table 6.3. Precision was evaluated by carrying out six independent sample preparation of a single lot of formulation. Sample was analyzed for five times after extracting the drug as mentioned in assay sample preparation of the experimental section. Low values of standard deviation denoted very good repeatability of the measurement. Thus, it was showing that the equipment used for the study was correct and hence the developed analytical method is highly repetitive. RSD of intraday precision was found to 0.435. For the interday precision a study carried out on the same day on two consecutive days indicated a RSD of 1.033. This indicates good method precision.

The stability of Rebamipide in standard and sample solutions containing determined by storing the solutions at ambient temperature (20±10°C). The solutions

were checked in triplicate after three successive days of storage and the data were compared with freshly prepared samples. In each case, it could be noticed that solutions were stable for 48 hrs, as during this time the results did not decrease below 98%. This denotes that Rebamipide is stable and standard and sample solutions for at least 2 days at ambient temperature. To check the degree of accuracy of the method, recovery studies were performed in triplicate by standard addition method at 50%, 100% and 150% to the proposed HPLC method. Results of recovery studies are shown in Table 6.6. The results showed good recoveries ranging from 99.0 to 100%. The mean recovery data obtained for each level as well as for all levels combined were within 2.0% of the label claim for the active substance with an R.S.D< 2.0%, which satisfied the acceptance criteria set for the study. The system suitability parameter like capacity factor, asymmetry factor, tailing factor and number of theoretical plates were also calculated. It was observed that all the values are within the limits (tailing factor < 2and number of theoretical plate's > 2000). The statistical evaluation of the proposed method was revealed its good linearity, reproducibility and its validation for different parameters and let us to the conclusion that it could be used for the rapid and reliable determination of Rebamipide in tablet formulation.

CONCLUSION

In this study, we developed an economical, simple, fast, and selective analytical method for analyzing Rebamipide in formulation using RP-HPLC. This method was proven to meet the validation requirements, which included selectivity, linearity, LOD and LOQ, accuracy, and precision. This method is considered suitable for quality control analysis in industry because it was found to be very sensitive and required only a small amount of sample.

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