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Method development and validation for the estimation of rebamipide in api form and marketed formulation

Santhoshillendula*, Konda Deepthi, Dr.K. Rajeswar Dutt

Department of Pharmaceutical Analysis, Nalanda College of Pharmacy, Cherlapally(v), Nalgonda (Dt), Telangana (St), India, 508001

***Corresponding Author: Santhoshillendula**
Email: santoshillendula@gmail.com

ABSTRACT

A new analytical, precise, accurate and rapid high performance liquid chromatographic method has been developed and validated for the estimation of Rebamipide in bulk form and marketed pharmaceutical dosage form. A Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm in isocratic mode, with mobile phase containing a mixture of Phosphate Buffer (0.02M): Acetonitrile in the ratio of 48:52 v/v (pH was adjusted to 2.80 with orthophosphoric acid) was used. The mobile phase was pumped at a flow rate of 1.0 ml/min and the eluents were monitored at 248 nm. The selected chromatographic conditions were found to effectively separate Rebamipide (Rt: 3.867min). The method was validated in terms of linearity, accuracy, precision, and specificity, limit of detection and limit of quantitation. Linearity for Rebamipide was found in the range of 30-70µg/ml. The percentage recoveries for Rebamipide ranged from 98%-120%. The limit of detection and the limit of quantitation for Rebamipide were found to be 0.09µg/ml and 0.027µg/ml respectively. The method was found to be robust and can be successfully used to determine the drug content of marketed formulations.

Key words: Rebamipide, RP-HPLC, Method Development, Validation, Precision, Accuracy.

INTRODUCTION

Rebamipide, an amino acid derivative of 2-(1H)-quinolinone, is used for mucosal protection, healing of gastroduodenal ulcers, and treatment of gastritis. It works by enhancing mucosal defense, scavenging free radicals, and temporarily activating genes encoding cyclooxygenase-2. Studies have shown that Rebamipide[14] can fight the damaging effects of NSAIDs on the GIT mucosa, and more recently, the small intestine, but not for naproxen-induced gastric damage. Rebamipide has been investigated for the treatment of Stomach Ulcer, Keratoconjunctivitis Sicca, and Gastric Adenoma and Early Gastric Cancer. Rebamipide is a quinolinone derivative with anti-ulcer and anti-inflammatory activities. Rebamipide[15] induces cyclooxygenase 2 (COX2) synthesis which results in an increase in endogenous prostaglandin synthesis in the gastric mucosa. This agent also inhibits H. pylori-induced production of tumor necrosis factor (TNF) alpha and subsequent inflammation of the gastric mucosa. In addition, Rebamipide scavenges oxygen-derived free radicals that potentially cause mucosal injury, and stimulates prostaglandin EP4 receptor gene expression followed by mucous secretion, thereby enhancing the gastric mucosal defense. The IUPAC Name of Rebamipide[16] is 2-[(4-chlorobenzoyl) amino]-3-(2-oxo-1H-quinolin-4-yl) propanoic acid. The Chemical Structure of Rebamipide as follows (Fig 1)

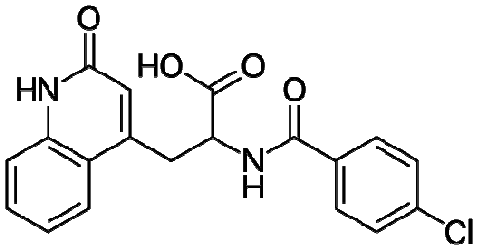

Fig-1: Chemical Structure of Rebamipide

The purpose of the present study is to establish a simple, sensitive, validated and inexpensive HPLC method for the determination of Rebamipide in pure form and in pharmaceutical dosage form.

EXPERIMENTAL

MATERIALS AND METHODS

INSTRUMENTS USED

Table-1:List of Instrument used

S. No.	Instruments/Equipments/Apparatus
1.	Waters HPLC with Empower2 Software with Isocratic with UV-Visible Detector.
2.	ELICO SL-159 UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry RP C_{18}, 5µm, 250mm x 4.6mm i.d.
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

CHEMICALS / REAGENTS USED

Table-2:List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
6.	Sodium hydroxide	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
7.	Hydrochloric acid	96%	A.R.	Sd fine-Chem ltd; Mumbai
8.	3% Hydrogen Peroxide	96%	A.R.	Sd fine-Chem ltd; Mumbai

Selection of wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent.(After optimization of all conditions) for UV analysis. Itscanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Rebamipide, so that the same wave number can be utilized in HPLC UV detector for estimating the Rebamipide.

Sample & Standard Preparation for the Analysis

25 mg of Rebamipide standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.3 ml of the above solution into a 10 ml volumetric flask and make up to volume with mobile phase.

Preparation of 0.02M Potassium dihydrogen orthophosphate Solution

About 2.72172grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC Grade water. The pH was adjusted to 2.80 with diluted orthophosphoric acid Solution.

Preparation of Mobile Phase

480mL (48%) of above Phosphate buffer solution and 520mL of HPLC Grade Acetonitrile (52%) were mixed well and degassed in ultrasonic water bath for 15 minutes. The resulted solution was filtered through 0.45 µm filter under vacuum filtration.

Method Validation

Validation[1,2]is a process of documenting and proving, analytical method provides analytical data, for the intended use. There are many reasons for the need to validate analytical procedures. To assuming the quality and achieving the quality control requirements, to achieve acceptance of the product by international agencies.

Accuracy
Recovery study

To determine the accuracy[3] of the planned technique, recovery studies were distributed by adds completely different amounts (80%, 100%, and 120%) of pure drug of Rebamipide were taken and extra to the pre-analysed formulation of concentration 30µg/ml. From that proportion recovery values were calculated. The results were shown in table-4.

Precision
1. Repeatability

The precision[4] of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Various precision levels are system or instrument precision, intermediate precision[5], repeatability, reproducibility[6].

2. Intermediate precision
2.1 intra-assay & inter-assay

The intra & inter day variation[7]of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Rebamipide revealed that the proposed method is precise.

Linearity & range

The calibration curve[8] showed good linearity[9] in the range of 0-70µg/ml, for Rebamipide (API) with correlation coefficient (r^2) of 0.999 (Fig-4). A typical calibration curve has the regression equation of y = 11266.x + 50416 for Rebamipide

Robustness

Influence of small changes in chromatographic conditions[10]such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & Acetonitrile content in mobile part (±2%) studied to work out the strength of the tactic also are in favour of (Table-8, nada RSD < 2%) the developed RP-HPLC technique for the analysis of Rebamipide (API).

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

Limit of detection[11]: is the lowest amount of an analyte in a sample which can be detected but not necessarily quantified as an exact value. Limit of quantitation[12] is the lowest concentration of an analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

System suitability parameter

System quality testing[13] is associate degree integral a part of several analytical procedures. The tests square measure supported the idea that the instrumentation, physics, associate degreeanalytical operations and samples to be analyzed represent an integral system that may be evaluated intrinsically. Following system quality check parameters were established. The info square measure shown in Table-9

Assay

Twenty pharmaceutical dosage forms were taken and the I.P. method was followed to work out the typical weight. On top of weighed tablets were finally pulverized and triturated well. A amount of powder cherish twenty five mg of medicine were transferred to twenty five cc meter flask, build and resolution was sonicated for quarter-hour, there once volume was created up to twenty five cc with same solvent. Then ten cc of the on top of resolution was diluted to a hundred cc with mobile part. The answer was filtered through a membrane filter (0.45±m) and sonicated to remove. The answer ready was injected in 5 replicates into the HPLC system and therefore the observations were recorded.

A duplicate injection of the quality resolution was conjointly injected into the HPLC system[14] and therefore the peak areas were recorded. The info square measure shown in Table-10.

Assay

Assay[15] % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{\text{a hundred}} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak space of drug obtained with check preparation
AS = Peak space of drug obtained with normal preparation
WS = Weight of operating normal taken in mg
WT = Weight of sample taken in mg
DS = Dilution of normal resolution
DT = Dilution of sample resolution
P = proportion purity of operating normal

Stability Studies

Acid Degradation

Following convention was entirely clung to for constrained corruption of Rebamipide Active Pharmaceutical Ingredient (API). The API (Rebamipide) was subjected to pressure conditions[16]in different approaches to watch the rate and degree of corruption that is probably going to happen over the span of capacity as well as after organization to body. This is one kind of quickened dependability contemplates that encourages us deciding the destiny of the medication that is probably going to occur after prolonged stretch of time stockpiling, inside a brief timeframe as contrast with the constant or long haul steadiness testing. The different debasement pathways[17]contemplated are Acid/corrosive hydrolysis, Alkali/fundamental hydrolysis, Thermal/warm Degradation, photolytic corruption/Degradation and oxidative Degradation/corruption.

Basic Hydrolysis

A precisely measured 10 mg of unadulterated medication was exchanged to a clean and dry round base flagon. 30 ml of 0.1N NaOH was added to it. & it was refluxed in a water bath at 600C for 4 hours. Allowed to cool to room temperature. The example was then killed utilizing 2N HCl arrangement and last volume of the example was made up to 100ml to plan 100 µg/ml arrangements. It was infused into the HPLC framework against a clear of portable stage in the wake of enhancing the versatile stage arrangements. This experiment was repeated several times using same concentration of NaOH such as 0.1N to observe its degradation profile. The chromatogram shown below is the degradation profile of Rebamipide in 0.1N NaOH.

Thermal Degradation

Precisely measured 10 mg of unadulterated medication was exchanged to a clean and dry round base carafe. 30 ml of HPLC water was added to it. Then, it was refluxed in a water bath at 600 c for 6 hours uninterruptedly. After the reflux was over, the drug became soluble and the mixture of drug & water was allowed to cool to room temperature. Last volume was made up to 100 ml with HPLC water to plan 100 µg/ml arrangements. It was infused into the HPLC framework against a clear of versatile stage/mobile phase.

Photolytic Degradation

Around 10 mg of unadulterated medication was taken in a clean and dry Petri dish. It was kept in an UV bureau at 254 nm wavelength for 24 hours without interference. Precisely measured 1 mg of the UV uncovered medication was exchanged to a clean and dry 10 ml volumetric cup. First the UV exposed drug was dissolved in methanol & made up to the mark with mobile phase to get 100 µg/ml solution. At long last this arrangement was infused into the HPLC framework against a clear of portable stage and chromatogram was gotten.

Oxidation with (3%) H2O2

Precisely measured 10 mg. of unadulterated medication was taken in a clean and dry 100 ml volumetric jar. 30 ml of 3% H2O2 and a little methanol was added to it to make it dissolvable and then kept in that capacity in dim for 24 hours. Last volume was made up to 100 ml. utilizing water to get ready 100 µg/ml arrangement. The above example was infused into the HPLC framework.

RESULTS AND DISCUSSION

Method development

Selection of wavelength:

While scanning the Rebamipide solution we observed the maxima at 248 nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450. The scanned UV spectrum is attached in the following page, (Fig 2)

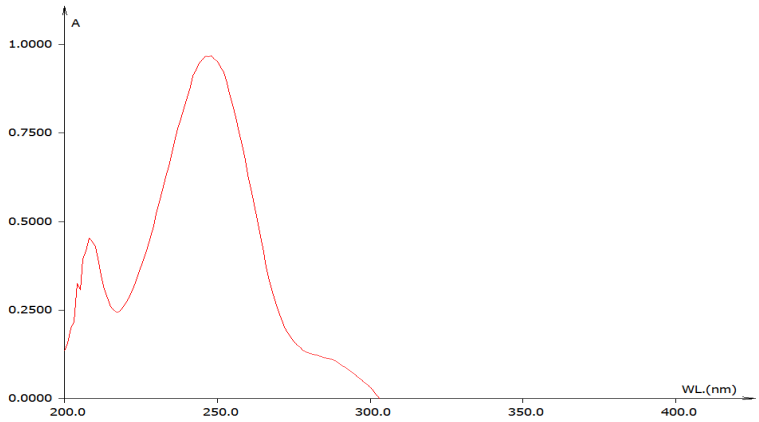

Fig-2: UV Spectrum for Rebamipide at 248 nm

Summary of optimised Chromatographic conditions

Table-3: Summary of optimised Chromatographic conditions

Mobile phase	Phosphate Buffer (0.02M): Acetonitrile = 48:52 (pH-2.80)
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	248 nm
Flow rate	1.0 ml/ min.
Run time	08 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Mode of Elution	Isocratic
Retention time	3.867 minutes

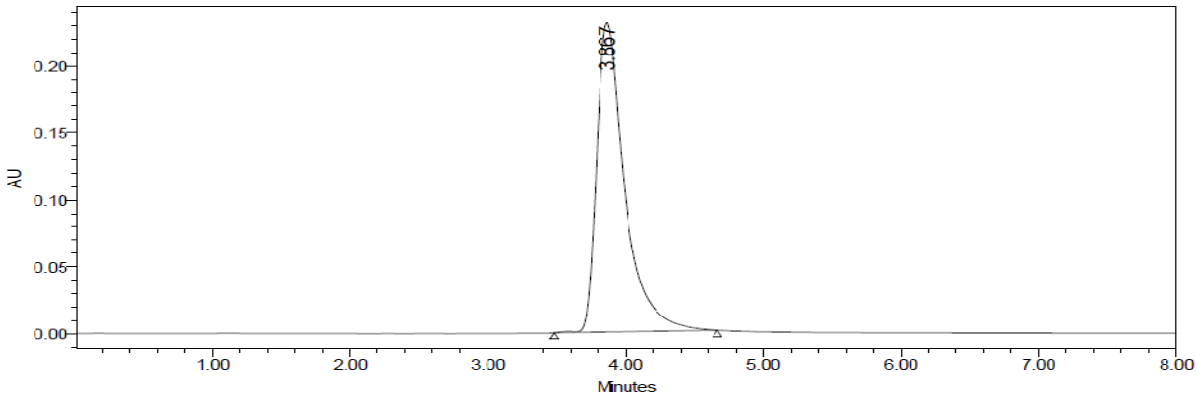

Fig-3: Chromatogram of Rebamipide in optimized chromatographic condition

Method validation

Accuracy

Accuracy results listed in Table 4 were found to be 100.230 ±0.47 % w/w for Rebamipide, which indicate high recovery of the method.

Table-4: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	%Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	40	40.141	502647	100.352	Mean= 100.3947% S.D. = 0.071319 % R.S.D.=0.071038
S_2 : 80 %	40	40.191	503214	100.477	
S_3 : 80 %	40	40.142	502656	100.355	
S_4 : 100 %	50	50.044	614215	100.088	Mean= 99.98533% S.D. = 0.183045 % R.S.D.=0.183071
S_5 : 100 %	50	49.887	612451	99.774	
S_6 : 100 %	50	50.047	614254	100.094	
S_7 : 120 %	60	60.192	728547	100.32	Mean= 100.311% S.D. = 0.408574 % R.S.D.=0.407308
S_8 : 120 %	60	59.939	725698	99.898	
S_9 : 120 %	60	60.429	731211	100.715	

Precision:

Repeatability

The exactitude of every technique was determined one by one from the height areas & retention times obtained by actual determination of six replicates of a set quantity of drug. Rebamipide (API). The % relative variance was calculated for Rebamipide square measure bestowed within the table-5.

Table-5: Repeatability Readings

HPLC Injection Replicates of Rebamipide	Retention Time (Minutes)	Peak Area
Replicate – 1	3.649	5674158
Replicate – 2	3.684	5654715
Replicate – 3	3.687	5665841
Replicate – 4	3.688	5654578
Replicate – 5	3.688	5652284
Replicate –6	3.687	5641487
Average		**5657177**
Standard Deviation		**11369.72**
% RSD		**0.200979**

Intermediate Precision

Intra-assay & Inter-assay

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Rebamipide revealed that the proposed method is precise. (Table 6)

Table-6: Results of intra-assay & inter-assay

Conc. ofRebamipide(API) (µg/ml)	Observed Conc. of Rebamipide (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
40	40.05	1.09	39.89	1.08
50	50.08	0.95	49.54	0.76
60	60.09	0.97	59.86	0.94

Linearity & Range

Calibration curve was constructed by injecting five different concentrations of Rebamipide.Results of the regression analysis and the coefficient of determination (r2) are listed in Table 1.The high coefficient of determination values i.e. 0.9997 for indicated good linearity between their peak areas (y) and standard drug concentrations (x,µg/ml) in the range

30-70µg/ml for Rebamipideand the obtained results are shown in Fig 4.

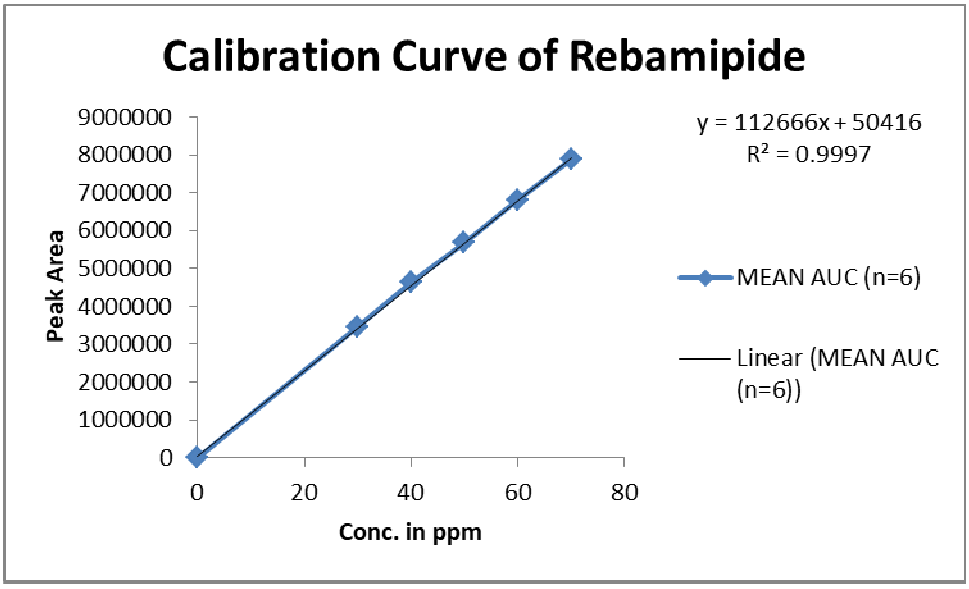

Fig-4:Calibration Curve of Rebamipide (API)

Table-7: Linearity Results

Conc.(µg/Ml)	Mean AUC (n=6)
0	0
30	3465974
40	4626478
50	5682284
60	6815478
70	7878721

Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis.The robustness of the method was evaluated by assaying the same sample under different analytical conditions deliberately changing from theoriginal condition. Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & Acetonitrile content in mobile part (±2%) studied to work out the strength of the tactic also are in favour of (Table-8, nada RSD < 2%) the developed RP-HPLC technique for the analysis of Rebamipide (API).The %RSD value of assay determined for the same sample under original conditions and robustness conditions was less than 2.0% indicating that the developed method was robust. (Table 8)

Table-8: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.56
Flow (0.9 ml/min)	0.87
Temperature (27^{0}C)	0.72
Temperature (23^{0}C)	0.53
Wavelength of Detection (257 nm)	0.61
Wavelength of detection (253 nm)	0.96

LOD & LOQ

The Minimum concentration level at whichthe analyte can be reliabledetected (LOD) &quantified (LOQ) were found to be 0.09 & 0.027 µg/ml respectively.

System Suitability Parameter

The system suitability test was performed to ensure that the complete testing system was suitable for the intended application. The parameters measured were peak area,

retention time, tailing factor and theoretical plates. In all measurements the peak area varied less than 2.0, the average retention time was 3.86 min, theoretical plateswere 4765 (more than 2000) and tailing factor was 1.42 (less than 2) for the Rebamipidepeaks as shown in Table 9 respectively. The proposed method offers high sensitivity and both the peaks can be detected accurately. In all the cases, the Rebamipidepeaks were well separated from the excipients. (Table 9)

Table-9: Knowledge of System quality Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	8.54
2	Asymmetry	T ≤ 2	Rebamipide =0.98
3	Theoretical plate	N > 2000	Rebamipide =4782
4	Tailing Factor	T<2	Rebamipide =1.49

Assay

Table-10: Recovery Data for estimation Rebamipide

Brand name of Rebamipide	Labelled amount of Drug (mg)	Mean	(± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
RebamipideTablets (100mg)	100mg		99.86 (± 0.682)	99.53 (±0.364)

Result & Discussion: The amount of drugs in Rebamipide Tabletwas found to be 99.86 (± 0.682) mg/tab for Rebamipide& % assay was 99.364 %.

Stability studies

The results of the stress studies indicated the specificity of the method that has been developed. Rebamipide was stable in photolytic and peroxide stress conditions. The result of forced degradation studies are given in the following table-11.

Table-11:Results of forced degradation studies of Rebamipide

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	98.76	1.24	100.0
Basic Hydrolysis (0.I M NaOH)	24Hrs.	98.63	1.37	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	93.98	6.02	100.0
UV (248nm)	24Hrs.	98.84	1.16	100.0
3 % Hydrogen Peroxide	24Hrs.	94.61	5.39	100.0

CONCLUSION

The proposed HPLC method was validated as per ICH guidelines and applied for the determination of Rebamipide in bulk form and marketed pharmaceutical formulations. The method was found to be accurate, precise, robust and specific. At the same time the chromatographic elution step is undertaken in a short time (< 5 min). No interference was seen from any components of pharmaceutical dosage form. In conclusion, the high sensitivity, good selectivity, accuracy and reproducibility of the proposed method are suitable for determination of Rebamipide in bulk form and marketed pharmaceutical formulations.

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