Stability Indicating RP-HPLC method development and validation for simultaneous quantification of antihistaminic & anti-asthmatic drug in bulk and tablet dosage form

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Abstract

This research describes the stability indicating RP-HPLC method in pharmaceutical tablet dosage forms for simultaneous estimation of Levocetirizine dihydrochloride (LEV) and Montelukast Sodium (MON). The proposed RP-HPLC method was developed using separation module Waters 2695 with PDA detector and chromatographic separation was performed at a flow rate of 1 mL / min using column Hypersil BDS C18 (250/4.6 mm, 5 μ m) with a run time of 10 min. The mobile phase consisted of a 40:60% v / v Phosphate buffer and acetonitrile, pH with orthophosphoric acid was adjusted to 7.0 and the eluents were scanned at 230 nm using a PDA detector. Retention times for LEV and MON were 3.06 min, and 6.76 min, respectively. A linearity response was observed with a concentration range of 12.56–37.68 μ g / mL for LEV and 23.78–71.20 μ g / mL for MON. Limit of detection and limit of quantification for LEV are 0.079 μ g/mL and 0.239 μ g/mL and for MON are 0.156 μ g/mL and 0.473 μ g/mL, respectively. The stability indicating method was developed by subjecting the drugs to stress conditions such as acid, base hydrolysis, oxidation, neutral, photo- and thermal degradation, and the degraded products produced from the samples were successfully solved.

Keywords: Levocetirizine dihydrochloride, Montelukast Sodium, RP-HPLC, stability indicating.

Introduction

Levocetirizine dihydrochloride (LEV), (2-[4-(R)-(4chlorophenyl) phenylmethyl]-1-piperazinyl]ethoxyacetate dihydrochloride) (fig. 1) is a third-generation non-sedative antihistamine, H1 receptor antagonist.¹⁻⁶ does block histamine receptors. This is used to treat several allergic reactions, i.e., allergic rhinitis, idiopathic urticaria, hay fever, etc.⁷ The half-dose form of LCTZ (2.5 mg) has been clinically proven to have equivalent antihistaminic effectiveness in the treatment of allergic rhinitis and chronic idiopathic urticaria to the normal amount of cetirizine (5.0 mg),^{2.8}

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Montelukast Sodium (MON) (1-[[(1R)-1-[3-(1E)-2-(7-chloro-2-quinolinyl) ethenyl] phenyl]-3-[2-(1-hydroxy-1-methylethyl) phenyl] -propyl] thio] methyl cyclopropaneacetic acid, monosodium salt (Fig. 2) is a white colored powder which is easily soluble in ethanol, methanol and water and is practically insoluble in acetonitrile. Montelukast Sodium molecular weight is 608.2 g/mol and C₃₅H₃₅ClNO₃S.Na is formula. Recent studies have shown that treatment with concomitant administration of anti-leukotriene and antihistamines for allergic rhinitis provides a significantly enhanced symptom relief compared to the mild improvement in rhinitis symptoms with each drug alone.^{5,9-11}

Fig. 1: Chemical structure of Levocetirizine dihydrochloride

Journal of Pharmaceutical and Biological Sciences, January-June, 2020;8(1):12-22



Fig. 2: Chemical structure of Montelukast sodium

The literature survey indicates that various methods have been published for evaluating LEV and MON in single-component formulations, but less methods are available for simultaneous evaluation of these two drugs in multicomponent dosage forms.^{3, 5,12-16} No analytical methods were reported in the presence of their degrading product for the simultaneous estimation of LEV and MON in bulk, and their combined dosage forms. Therefore, the purpose of this work was to describe a simple, accurate, repeatable and time mitigation RP-HPLC method for the simultaneous determination of LEV and MON in the tablet dosage form and validation of the same as according to ICH guidelines.¹⁷⁻¹⁹

Materials and Methodologies Chemical and reagents

The MON and LEV APIs are collected from the company Emcure Pharmaceuticals Ltd. Pune, India as a gift sample. Acetonitrile grade HPLC shall be obtained from Merck (Mumbai, India), HPLC grade Water (Milli Q) for a complete chemical analysis (AR grade). Pharmaceutical tablet dosage form containing 10 mg + 5 mg of Nukast-10 was purchased from local pharmacy.

Instrumentation

The HPLC experiment is performed on a Waters Alliance 2690 separation module with a Waters 2996 photodiode array detector using an Auto Sampler. Data collection and processing have been prepared using EMPOWER PDA 2 software. The analytical column used for the separation was Hypersil BDS C18, 250 mm \times 4.6 mm Column, 5 µm, Other equipment's used were ultra-sonicator (Equitron), Single Pan Electronic Balance (Startorious) PH Meter (LABINDIA).

Preparing solutions

Buffer

Mix 2.8 gm of Disodium hydrogen orthophosphate dihydrate in 1000 ml of water, adjust pH-7.0 with OPA.

Mobile phase

Mix acetonitrile and phosphate buffer with a pH-7.0 ratio of 60:40. Filter through 0.45μ membrane filter paper.

Diluents

Mobile phase used as diluent.

Preparing a standard solution

Solution A

Weigh exactly about 25 mg of Levocetirizine dihydrochloride in a 50 ml volumetric flask. Add 35 ml of diluents, dissolve to sonicate and dilute to volume with the diluents. Transfer 1 ml of the above solution to 20 ml with diluents to make a 25 ppm solution.

Solution B

Weigh exactly about 50 mg of Montelukast Sodium in a 50 ml volumetric flask. Add 35 ml of diluents, dissolve to sonicate and dilute to volume with the diluents. Transfer 1 ml of the above solution to 20 ml with diluents to make 50 ppm of the solution.

Conditions of chromatography

The HPLC experiment is performed on the Waters Alliance 2690 Separation Module with the Waters 2996 Photodiode Array Detector using the Auto Sampler. Data collection and analysis was carried out using the EMPOWER PDA 2 software. The analytical column used for the separation was Hypersil BDS C18, 250 mm \times 4.6 mm, 5 µm; Other equipment used were ultrasonicator (Equitron), single pan electronic balance (Startorious) PH meter (LABINDIA). Characteristic chromatography of LEV and MON was shown in Fig. 3 and optimized chromatographic conditions as shown in Table 1.



Fig 3: Typical chromatogram of LEV and MON

Table 1: (Optimized	conditions	of chro	matography
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Column	Hypersil BDS C_{18} ,
	250 mm \times 4.6 mm, 5 μ m
Mobile phase	Acetonitrile : Phosphate buffer
	pH-7.0 (60:40)
Flow rate	1.0 ml/min
Column temperature	30°C
Injection Volume	10 μL
Detection	230 nm
Wavelength	
Run time	10 min
Retention time	3.06 (LEV), 6.76 (MON) min

Wavelength selection

In the Fig. 4 LEV and MON UV spectrum, wavelength 230 nm showing isobestic radiation, was selected.



Fig. 4: UV Spectrum of LEV and MON

Table 2: Marketed Formulation Analysis Results
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Development of the method

To saturate the column, the mobile phase was pumped for about 30 minutes so that the base line could be corrected. Standard calibration lines have been designed for each drug. A series of aliquots were prepared from the above-mentioned stock solutions using diluents to obtain concentrations of 12.56-37.68 μ g / ml for LEV, 23.78–71.20 μ g / ml for MON. Inject each concentration 6 times into the chromatography system. Each time the peak is and the retention time for both drugs has been recorded separately. Calibration curves are constructed as individual for both drugs by taking the average peak area of the Y-axis and the concentration of the X-axis Regression equations was derived from the calibration curves. These regression equations are used in the formulation to measure the content of the product. LEV and MON measured in tablet dosage form.

Weigh 20 tablets and crush to powder, then add powder equivalent to 25 mg of LEV and 50 mg of MON to a 100 ml volumetric flask. Add 70 ml of diluents, sonicate for 10 min to dissolve the active ingredients and dilute to volume with the diluents. Filter through the Whatman filter paper no. 41. Then dilute 5 ml–50 ml with the diluents. Filter with 0.45μ Nylon syringe filter. This solution was estimated using the abovedeveloped method. The assay procedure was repeated 6 times (n = 6) the drug content was estimated using the above calculated regression equation; the results of the tablet dosage form are shown in Table 2.

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Name of the	Brand	Label claim	Test concentration	Mean amount estimated	%	%RSD
compound	name	(mg)	(µg/ml)	(µg/ml) (n=6)	Assay	
LEV	Nukast-10	5mg	25	24.88	99.55	0.43
MON		10mg	50	49.18	98.37	0.57

Validation of method

The method described has been validated for linearity, accuracy, detection limit, precision and robustness, as set out in the ICH guidelines.²⁰

Linearity

The linearity of the method was determined by the preparation of six different concentrations of LEV and MON within the 12.56–37.68 μ g / ml and 23.78–71.20 μ g / ml concentration ranges. Every solution was prepared in a triplicate. Plotting peak area against concentration was used to obtain the calibration curve. Linearity was tested over the same concentration range over three consecutive days and results were obtained. Results have been shown in Table 3a-3c and graphs shown in Figure 5 A-B.

Table 3a: Linearity studies of LEV.

S. No.	Concentration (µg/ml)	Average peak area
1.	0	0
2.	12.56	251813
3.	18.84	368600
4.	25.12	495138
5.	31.40	616357
6.	37.68	744210

Table 3b: Linearity studies of MON.

S. No.	Concentration (µg/ml)	Average peak area
1.	0	0
2.	23.73	680965
3.	35.60	997397
4.	47.47	1338187
5.	59.34	1667722
6.	71.20	2012095

Table 3c: LEV and MON optical characteristics

Parameters	LEV	MON
Linearity range (µg/ml)	12.56–37.68	23.73–71.20
Regression equation	y = 19624.05x + 2203.20	y = 28082.68x + 6239.20
Slope	19624.05	28082.68
Intercept	2203.20	6239.20
Correlation coefficient (R ²)	0.9998	0.9998



Fig. 5: Linearity of the plot A) Levocetirizine dihydrochloride B) Montelukast sodium.

Accuracy, as recovery

The accuracy of the method was determined at three different concentration levels: 50 %, 100 %, and 150 % of the known amount of drug analyte, and a % of recovery was calculated. And the results have been shown in Table 4.

Drug	% Recovery	Pre-analyzed	Known amount	Amounts	%	%RSD
	level	conc (µg/ml)	added (µg/ml)	found (µg/ml)	Recovery	
LEV	50%	31.30	15.66	15.81	100.96	0.10
	100%	31.30	31.30	31.09	99.33	0.13
	150%	31.30	46.94	46.69	99.47	0.15
MON	50%	62.50	31.25	31.26	100.03	0.26
	100%	62.50	62.51	61.33	98.11	0.33
	150%	62.50	93.75	92.57	98.74	0.40

Table 4: LEV and MON recovery studies.

Precision

Precision of the method (Repeatability)

The precision method is determined by injecting six working standard solutions and six sample injections. The areas of all injections were taken and the standard deviation, % relative standard deviation (RSD), % assay was calculated.

Intermediate precision

The intermediate precision was determined by injecting six working standard solutions and six sample injections on different days by different operators or by different instruments. All injection areas were taken and the standard deviation, % relative standard deviation (RSD), % assay was calculated. The results were shown in Tables 5a and b.

S. No	LEV	MON
1	502621	1364588
2	501951	1361227
3	501602	1365023
4	501735	1365080
5	499351	1361537
6	501702	1365123
Mean	501494	1363763
Standard deviation	1112.2	1856.8
%RSD	0.22	0.14

Table 5a: Method Precision studies for LEV and MON.

Table 5b: System Precision studies for LEV and MON

S. No	LEV	MON
1	502437	1354588
2	499864	1361277
3	500123	1375023
4	499475	1369080
5	498537	1341567
6	501679	1362123
Mean	500353	1360605
Standard deviation	1446.5	11681.2
%RSD	0.28	0.85

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD

It is the lowest amount of analyte in the sample that can be detected but not necessarily quantified as an exact value in the experimental conclusions. The detection limit is usually expressed as an analytical concentration. The standard deviation and the slope response and the results obtained. LOD = 3.3*S / NS / N.

LOQ

The quantitative limit of the analytical procedure is the lowest amount of the sample analyte that can be quantitatively determined with appropriate precision and accuracy. The standard deviation and the slope response and the results obtained. And the results have been shown in Table 6. LOQ = 10 S / N.

	LEV	MON
LOD (µg/ml)	0.079	0.156
LOQ (µg/ml)	0.239	0.473

Table 6: LOD and LOQ for LEV and MON.

Parameters of system suitability

In order to assess system suitability, six replicates of working standards samples of Ledipasvir and Sofosbuvir were injected and parameters such as plate number(N), tailing factor(K), resolution, relative retention time and peak sample asymmetry were studied. The results have been tabulated in Table 7.

Parameter	LEV	MON	Criteria for acceptance
Retention time	3.03	6.79	For information
Plate count	37873	71635	NLT 2000
Tailing factor	1.25	1.17	NMT 2
Resolution	5.49	5.14	NLT 1.5

Table 7: System suitability parameters for LEV and MON

Robustness

The robustness of the assay method was calculated by adding minor chromatographic changes, including wavelength (228 nm–232 nm), flow rate (0.9 and 1.1 mL / min) and pH (+ 0.2 % to -0.2 %). The findings have been tabulated in Table 8.

Table 8: Robustness studies for LEV and MON

Method parameters	Conditions	Retentio	n Time (R _t)	A	rea	%Rec	covery
		LEV	MON	LEV	MON	LEV	MON
Flow +	+0.1	2.97	6.67	560587	1592893	101.56	100.87
Flow –	-0.1	3.84	8.60	458881	1304626	101.66	101.01
pH +	+0.2	2.52	5.64	510992	1456552	102.41	101.75
pH–	-0.2	3.76	8.84	508326	1442763	101.86	101.27
Wavelength +	+2	3.01	6.74	494758	1536167	101.74	101.06
Wavelength –	-2	2.87	6.43	498481	1363403	101.84	101.17

Selectivity and specificity

Specificity is the level to which the technique applies to a single analyte, and is tested for any interfering peaks in each study by analysing blank matrix samples. The specificity of the method was evaluated for interference due to the presence of any other placebos. Two separate samples were injected and tested with placebos, respectively. The HPLC chromatograms reported for the drug matrix (drug mixture and placebos) showed nearly no interfering peaks with time ranges in retention.

Forced degrading studies

Forced drug formulation degradation studies have been conducted to treat drug samples under stress-induced conditions such as acid and base hydrolysis, oxidation, photo-and thermal degradation and interference of degraded products. These studies help to determine the inherent stability of the effective molecules in the drug product and the potential degradation products.^{21, 22}

Preparing of sample stock solution

For forced degradation studies, the powder sample was carefully weighed equivalent to 25 mg of LEV and 50 mg of MON and transferred to a 100 ml volumetric flask containing 70 ml of diluent, dissolving sonicate for 10 min.

Studies on Acid degradation (0.1 M HCL)

5 ml of test stock solution was taken in a 50 ml volumetric flask, 5 ml of 0.1 M HCl was added and heated to 80 $^{\circ}$ C for 1 hour in a water bath. The flask was removed and allowed to cool at room temperature. Add 5 mL of 0.1 M NaOH to neutralize the solution and dilute to volume with diluents and mixed solution.10 mL of solution was injected into the system and chromatograms were recorded to assess the stability of the sample.

Studies on Alkali degradation (0.1 M NaOH)

From the test stock solution 5 ml was taken in 50 ml volumetric flask, add 5 ml of 0.1 M NaOH and heated at 80 °C for 1 hour on a water bath. The flask was removed from the water bath and allowed to cool at room temperature. Add 5 ml of 0.1 M HCL to neutralize the solution and diluted to volume with diluents and mixed.10 μ l solution were injected in to the system and the chromatograms were recorded to assess the stability of sample.

Studies on Peroxide degradation (0.3% of H_2O_2)

In a 50 ml volumetric flask, 5 ml of 0.3 percent H2O2 was added to the test stock solution and heated to 80 $^{\circ}$ C for 1 hour in a water bath. The flask was removed

from the water bath and cooled at room temperature and diluted to volume with diluents and mixed solution.10 μ l of solution was injected into the system and chromatograms were collected to evaluate the stability of the sample.

Studies on Hydrolysis degradation

5 ml of test solution was taken in a 50 ml volumetric flask and 5 ml of water was added and heat to 80 $^{\circ}$ C for 1 hour in a water bath. The flask was withdrawn from the water bath and cooled at room temp and diluted to volume with diluents and mixed. 10 µl solution was injected into the system and the sample stability was measured using chromatograms.

Studies on Thermal degradation (80°C /48 hrs)

To test dry heat degradation, the drug sample was put in oven at 80 ° C for 48 hrs. Weight powder equal to 25 mg of LEV added to 100 ml of volumetric flask for HPLC analysis, add 30 % diluent, sonicate for 15 minutes. Filter the solution through Whatman filter paper no. 41, 5.0 ml filtrate pipette into 50 ml of vol. Flask, and finally render to diluent level. 10 μ l solutions was injected into the system, and chromatography was reported to evaluate the sample stability.

Studies on Photolytic degradation

The drug's photochemical stability was also tested by 48 hrs of exposing product sample powder to UV Radiation. Weight powder equal to 25 mg of LEV added to 100 ml of volumetric flask for HPLC analysis, add 30 % diluent, sonicate for 15 minutes. Filter the solution through Whatman filter paper no. 41, 5.0 ml filtrate pipette into 50 ml of vol. Flask, and finally render to diluent level. 10μ l solutions were injected into the system, and a chromatogram was reported to determine the sample stability.

At each point of the above-mentioned studies the purity of the drug peaks was tested using the peak purity test. And the results are expressed in Table 7 and Fig. 7. 10 μ l solution was injected into the system and the sample stability was measured using chromatograms.

Results and Discussion The chromatographic conditions are optimized

Most HPLC methods documented using the C-8 or C-18 columns to report. Complex compositions of the mobile phases are also used. Therefore, attempts on the widely used Hypersil BDS C18 column were based on the development of a Simple and Effective method with good resolution. In order to achieve a reasonable separation between drugs and degraded products, different conceptual modifications were attempted. Such improvements included change in mobile phase composition in isocratic elution, as well as gradient modes on different HPLC columns.

The chromatographic optimization conditions Fig 3. The best peak form and maximum separation was achieved with the mobile phase composition of Acetonitrile and Phosphate buffer pH-7.0 (60:40), peak symmetry and reproducibility was obtained with Hypersil BDS C18, 250 mm and 4.6 mm, 5 μ m column. The optimum wavelength for analyte detection was found to be 230 nm, with a flow rate of 1.0 ml / min providing optimal separation and peak symmetry. LEV chromatography and MON Fig 3. And Table 1 displays optimized chromatographic state.

Accuracy and precision

By spiking previously analyzed test solution with additional Placebo at three separate concentration points Table 4, accuracy as recovery was evaluated. Recovery of previously tested drug concentration added test solution was found to be 100.08 % for LEV and 98.9 % for MON with RSD value less than 1 % suggesting that the proposed approach is suitable for simultaneous estimation for both drugs from their combination drug products in the presence of their degradation products. The low RSD values suggest the repeatability and reproducibility of Tables 5a and 5b of the process.

Linearity, LOD and LOQ

The calibration plot was linear across the examined concentration range (12.56–37.68 μ g / ml) for LEV, (23.78–71.20 μ g / ml) for MON, respectively. The average correlation coefficient R2 = 0.999 was derived from the regression analysis to both drugs with % RSD

values as well as 2.0 around the concentration ranges tested. The LOD which generated the necessary accuracy and precision was found to be $0.079\mu g$ / ml LEV and $0.156\mu g$ / ml MON. The corresponding % of RSD values in Table 3c was 0.1.00 %. The LOQ for LEV and MON was $0.239\mu g$ / ml, $0.473\mu g$ / ml, respectively. The findings of the Regression suggest that the process was linear in the concentration range tested and can be used to detect and measure LEV and MON within a very large range of concentrations. Illustration. 5A – B show LEV and MON linearity graphs, respectively.

Selectivity and specificity

In each study the specificity is tested by analyzing blank and placebo samples for any interfering peaks. Owing to the existence of some other excipient the precision of the approach was assessed with respect to intervention. The figures indicate clearly distinguishing the chosen products. Fig. 6a and b displays a chromatogram of a blank and working sample solution for placebo. No interfering peaks occurred during LEV and MON retention times.

Robustness

Robustness Results Table 8. The elution order and resolution were not substantially impacted for all materials. RSD of peak areas was found to be far below the 2.0 % mark.

System suitability

The parameters of suitability for the system were found to fall within acceptance criteria. Good peak with resolution between two drugs is > 1.5, asymmetric factor < 2 indicates stronger separation of the drugs. A number of theoretical plates, tailing factor, resolution, retention time, and area were the parameters measured for device suitability. Table 7 displays progress.

Degradation studies

The results of the stability studies were shown in Table 9 and Fig 6 a-f.

Table 9: LEC-MON stability studies

Stress conditions	LEV	MON	
	% degradation	% degradation	
Acid/0.1 M HCL/80°C reflux/3 hrs	10.62	12.27	
Basic/0.1 M NaOH/80°C reflux/3 hrs	5.11	5.90	
Oxidizing/0.3% H ₂ O ₂ /cool at RT/3 hrs	3.59	1.02	
Hydrolysis/Water/80°C/3 hrs	8.75	2.77	
Thermal/80°C/48 hrs	0.75	2.63	
Photolysis/UV light	5.91	27.84	



Fig. 6: (a-f): Degraded chromatograms – (a) Acid degradation (b) Alkali degradation (c) Oxidation degradation (d)Hydrolytic degradation (e) Thermal degradation (f) Photolytic degradation.

Acid hydrolysis (Fig. 6a)

10.62% of LEV and 12.27% of MON were degraded upon completion of acid degradation studies.

Base hydrolysis (Fig. 6b)

5.11% of LEV and 5.90% of MON were degraded upon completion of base degradation studies.

Peroxide hydrolysis (Fig. 6c)

3.59% of LEV and 1.02 % of MON were degraded upon completion of peroxide degradation studies.

Hydrolytic degradation (Fig. 6d)

8.75% of LEV and 2.77% of MON were degraded upon completion of hydrolytic degradation studies.

Thermal degradation (Fig. 6e)

0.75% of LEV and 2.63% of MON were degraded upon completion of thermal degradation studies.

Photolytic degradation (Fig. 6f)

5.91% of LEV and 27.84% of MON were degraded upon completion of photolytic degradation studies.

Statistical analysis showed that the method proposed was simple, accurate and reliable when compared to the reported method.³

Conclusion

A simple, accurate, commercial and time depleting RP-HPLC method was developed by using Hypersil BDS C18 (250×4.6 mm; 5µm) column, at 30°C temperature using mobile phase Disodium hydrogen orthophosphate dihydrate as Buffer (pH 7.0 with OPA) : Acetonitrile in 40:60 % v/v proportion at 1 ml/min flow rate. Detection was carried out at 230 nm found to give sharp, well defined peaks with good symmetry $t_R 3.06 \pm 0.5$ min and 6.76 ± 0.5 min for LEV and MON respectively. The method was validated with respect to various parameters such as linearity, range, precision, accuracy, robustness etc. The linear calibration curve for LEV was obtained at concentration range 12.56-37.68 µg/ml with r^2 0.9998. For MON 23.73-71.20 µg/ml with r^2 0.9998. The proposed method was employed for the forced degradation studies of LEV and MON. The degradation pathways under different conditions studied. For LEV and MON show 30-40% degradation for oxidation (3% H₂O₂ for 1 hrs), 20-25% degradation for U.V-light (48 hrs). So it can be concluded that both drugs were extremely labile to oxidation as well as photolytic condition. Found to be stable for base, neutral and dry heat degradation as less than 10% of drugs were degraded. 10-15% LEV and MON was degraded in acidic condition, So it is concluded that both drugs were unstable to acid conditions.

The developed HPLC method is simple, accurate, time depleting, reproducible and stability indicating for quantitative analysis of LEV and MON in pharmaceutical dosage form, without any interference from the excipients and in the presence of its acidic, alkaline, oxidative, thermal, neutral and photolytic degradation products. The chromatographic method was validated in accordance with the ICH guidelines. Statistical tests indicate that the proposed HPLC method reduces the duration of the analysis and appears to be equally suitable for routine determination of LEV and MON in pharmaceutical dosage form in quality control laboratories where economy and time are essential. This study is typical example of development of stability indicating assay, it is one of the rare studies where forced decomposition was done under all different suggested conditions and the degradation products were resolved. As the method separates the drug from its degradation products, it can be employed as a stability indicating method

Acknowledgment

The author are extremely thankful to Dr. Vikram S. Gharge, Associate Director of Research and Development, Emcure Pharmaceuticals Ltd. Bhosari, Pune (M.S.), India for the provision of facilities and gift samples for research purposes. Also thankful to Principal, H. R. Patel Institute of Pharmaceutical Education and Research. Shirpur, Dhule (M.S), India.

Source of Funding

None.

Conflict of Interest

None.

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How to cite: Sonawane JK, Patil DA, Jadhav BS, Jadhav SL, Patil PB. Stability Indicating RP-HPLC method development and validation for simultaneous quantification of antihistaminic & anti-asthmatic drug in bulk and tablet dosage form. *J Pharm Biol Sci* 2020;8(1):12-22.