Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 13[1] December 2023 :129-140

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Development and Validation of a Stability Indicating Green Analytical Method for The Simultaneous Estimation of Ambroxol and Loratadine in Bulk and Pharmaceutical Dosage forms by RP-HPLC

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ABSTRACT
The reversed-phased high-pressure liquid chromatography (RP-HPLC) method for the estimation of Ambroxol (Amb) and Loratadine (Lora) in bulk drugs and pharmaceutical dosage forms has been developed, optimized, and validated. The flow rate of 1 ml/min was optimized and an injection volume of 20 µL, the validated method evaluation was carried out using a Shimadzu RP-HPLC system, which was equipped with a symmetry column and detected using PDA (Photo diode array detector) detector at a wavelength of 246 nm. The mobile phase consisted of 0.1M potassium dihydrogen phosphate buffer with a pH adjusted to 6.2 using 0.1% orthophosphoric acid and acetonitrile in a 55:45, v/v ratio. Amb and Lora were found to have a retention time of 5.894 minutes, 3.202 minutes. The method was then validated using International Conference on Harmonization (ICH) Q2 R1guidelines. The method has been validated for linearity, precision, accuracy and specificity and method was showing that linearity in a concentration of range of 15-90µg/ml for Amb and 1.25-7.5 µg/ml for loratadine with correlation coefficients of r^2=0.999. Percentage recovery values were found to be 99.40%-99.42% for Amb and 99.11%-99.26% for Lora respectively. %RSD values of precision were determined to be 0.33% and 0.46%, correspondingly. The estimated limits of detection (LOD) for Amb and Lora were determined to be 0.03 µg/ml and 0.7µg/ml, respectively, while the limits of quantification (LOQ) for Amb and Lora 2.18 µg/ml and 0.09 µg/ml, respectively. The developed method was then performed force degradation and stability studies along with the green assessment. After running the proposed approach using tools likes the AGREE software, it was determined to have a lower greenness score (0.72). According to the results, the suggested method is appropriate for Ambroxol and Loratidine estimation in combined dosage form along with the stability studies with green assessment and method can be reliably used in routine labs and quality control laboratories for determination of the content of both the drugs. The proposed method was accurate, precise, robust, economical, and reproducible.
Keywords: *Ambroxol, Loratadine, ICH guidelines, retention time, green assessment.*

Received 19.10.2023 Revised 20.10.2023 Accepted 04.12.2023

INTRODUCTION

The alkaloid vasicine derivative ambroxol hydrochloride is derived from the plant Vasaka-*Adhatoda vasica* and is a powerful mucolytic drug that can induce thin abundant bronchial discharge, which helps with expectoration [1-3]. Through its mucolytic and mucokinetic actions, ambroxol hydrochloride depolymerizes lengthy mucopolysaccharide chains, causing them to fragment, and allowing for the expectoration of excessive secretions. Because it inhibits the release of harmful mediators and free oxygen radicals by phagocytosis, ambroxol hydrochloride also functions as a tissue protector [4-6]. Loratadine is an allergist's go-to medication since it blocks second-generation peripheral histamine H1-receptors. Atypical antipsychotic quetiapine is a distant relative, and it shares structural similarities with tricyclic antidepressants like imipramine. It wasn't until 1993 that loratadine hit the market, despite its 1981 discovery. The medicine is marketed for its non-sedating characteristics, is available as a generic version, and is on the List of Essential Medicines by the World Health Organization. An alternative formulation, pseudoephedrine/loratadine, is available; it is a decongestant. As a specific inverse agonist of peripheral histamine H1-receptors, the tricyclic antihistamine loratadine alleviates allergic reactions. A lot of the symptoms of an allergic reaction are caused by histamine [7-11], structures of Amb and Lora as shown in Figure 1.

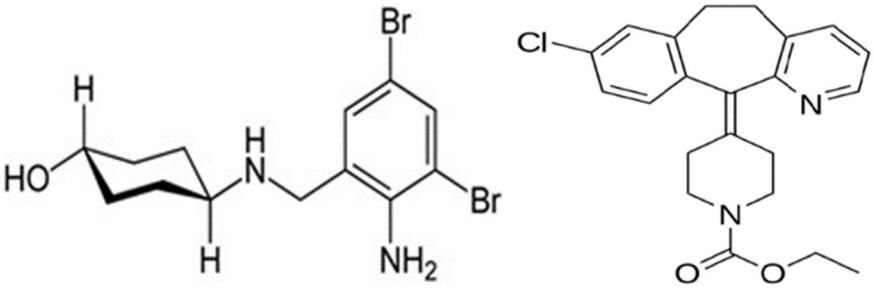


Fig. 1. Structure of Ambroxol and Loratadine

Pharmaceutical companies and clinical research laboratories regularly do quantitative analysis of pharmaceutically active components, whether they are in pure forms or drug formulations. In recent times, researchers have shown a preference for adopting the principles of green analytical chemistry (GAC). The main objective of GAC is to reduce or eliminate the negative effects of chemical processes on the environment and promote sustainability at the molecular level. It is not unexpected that many industrial sectors presently prioritize the environmental friendliness of chemical processes due to the significant benefits they offer, such as cost reduction, increased production, and savings in time and resources. In the 1990s, Paul Anastas and John Warner authored the inaugural green chemistry manual, which presented a series of 12 green chemistry principles. These principles focused on the elimination or end of the use of harmful solvents in chemical processes and evaluations, as well as the prevention of residue formation resulting from these processes. Subsequently, Paul Anastas emphasized the necessity of integrating these 12 principles to mitigate the environmental impact of new procedures and analytical tools. Consequently, the development of analytical methods is currently one of the most actively researched and studied issues in the field of green chemistry. In 2013, Galuszka, Migaszewski, and Namienski revised the 12 principles of green chemistry to align more closely with green analytical chemistry. The study describes the 12 concepts of green analytical chemistry as significant. Greenness assessment methodologies, including the national environmental method index (NEMI), the analytical eco-scale assessment (ESA), AGREE—Analytical Greenness Metric Approach, and the green analytical process index (GAPI), by the 12 principles of green chemistry. The NEMI approach, while simple and straightforward to use, is less accurate than other methods and is considered the earliest. The ESA tool utilizes numerical final results to determine the most environmentally friendly method employed. Furthermore, it is uncomplicated and more precise compared to the NEMI. The GAPI and AGREE is a recently developed and comprehensive method that offers a more accurate assessment of greenness. This study applies AGREE Methodologies.

During all stages of pharmaceutical development, analytical procedures are necessary to characterize the drug component and the composition of the drug product. An analytical technique must be validated by the guidelines provided by the International Conference on Harmonisation once it has been created for its intended purpose. According to the ICH guideline Q1A on Stability Testing of New Drug Substances and Products, stability-indicating testing procedures must be validated before testing features that are expected to change during storage and impact quality, safety, and/or efficacy can be conducted. Drug analysis improvement increasing with the use of analytical technique called high-performance liquid chromatography. It is perfect for analyzing a wide range of drugs in pharmaceutical dosage forms and biological fluids due to its ease of use, high sensitivity, and specificity.

Thus, the current study's goal is to create stability-indicating analytical technique for drug quantification in their formulations. The first objective deals with the optimization of chromatographic conditions and validation of the stability indicating analytical method for parameters such as specificity, precision, accuracy, linearity and range, LOD, LOQ, Robustness, and stability studies. The second part includes its Greenness evaluation by Agree software.

An extensive literature survey revealed that many analytical methods have been reported for the quantitative analysis of ambro & Lora alone and also in combination with other drugs in pure drugs and marketed formulations like UV, RP-HPLC [12-20]. The main goal of the green analytical chemistry is to take into the consideration the amount and toxicity of reagents can reducing the environmental impact of the activities of analytical chemistry. Stability indicating green reverse phase method has not been reported in the literature for the analysis of Amb and Lora, therefore the aim of the present study was to develop and validate a simple, cost effective, rapid, selective, precise, facile, reproducible, accurate stability indicating green RP-HPLC method for the analysis of Amb and Lora in bulk and marketed formulations.

MATERIAL AND METHODS

Using a Waters Alliance 2695 model HPLC system with a 2998 model PDA detector and Empower 2 software for processing and data collection, an isocratic RP-HPLC technique was used. The stationary phase was a 150 mm x 4.6 mm ID, 5 µm Symmetry C_{18} HPLC Column. The investigation makes use of an analytical balance, an ultrasonic bath sonicator, and Whatman filter paper No. 41.

Reagents used

Leeford Pharmaceuticals in India provided the ambroxol and loratadine. We bought HPLC-grade acetonitrile from Merck Specialties Private Limited in Mumbai, India. Water, potassium dihydrogen phosphate, and orthophosphoric acid were purchased from Rankem Ltd. in India and were of HPLC grade. India's Cadila Pharmaceuticals Limited is the source of the Lorfast-AM Tablets®.

Preparation of mobile phase

A precisely measured 0.1M potassium dihydrogen phosphate was made, and its pH was brought to 6.2 using 0.1% orthophosphoric acid. It was then put into a 1000 mL volumetric flask and combined with HPLC-grade acetonitrile in a 55:45 v/v ratio. Ultimately, a 0.45 µm membrane filter was used for filtration, and ultrasonication was employed to eliminate any residual gas.

Preparation of Ambroxol and Loratadine mixed standard drug stock solutions

To get a concentration of 600µg/mL of Ambroxol and 50µg/ml of Loratadine, 60 mg of Ambroxol and 5 mg of Loratadine were dissolved in 100 mL of the mobile phase and then thoroughly dissolved using sonication in a 100 mL volumetric flask.

Preparation of linearity solutions

The concentration of 15, 30, 45, 60, 75, and 90 µg/mL of Ambroxol and 1.25, 2.5, 3.75, 5, 6.5, and 7.5µg/mL of Loratadine was prepared for linearity respectively. Before being injected into the HPLC system, all of the aforementioned solutions were filtered using a 0.45 µm nylon membrane filter.

Preparation of sample solution

Each tablet of Lorfast-AM Tablets® contains 60 mg of Ambroxol and 5 mg of Loratadine was taken and weighed. Twenty Lorfast-AM Tablets® were weighed, then crushed, and combined in a mortar and pestle to create a powder. A precisely weighed amount of powder, equal to 60 mg of Ambroxol and 5 mg of Loratadine, was put into a 100 mL volumetric flask that had been cleaned and dried. After adding the mobile phase and fully dissolving it with sonication, the mixture was filtered through a 0.45 µm nylon membrane filter. The volume was then adjusted with the same mobile phase to obtain concentrations of 50µg of Loratadine and 600µg of Ambroxol per millilitre. To get 60µg/mL of ambroxol and 5µg/mL of loratadine solution, From the previously mentioned solution, 1 mL was pipetted out, transferred to a 10 mL volumetric flask, and diluted with the mobile phase until the desired level was reached.

Standard and sample solution for assay studies

Six injections of an aliquot of 20 µL of the standard and sample solutions, each containing 60 µg/mL of ambroxol and 5 µg/ml of loratadine solution, were injected in HPLC shown in Table 1.

Results for assay studies

Table 1. Assay of Ambroxol and Loratadine

Drug	Lorfast-AM Tablets® (mg)	Amount found*(mg)	Label claim %
Ambroxol	60	59.88	99.8
Loratadine	5	5.005	100.1

RESULTS AND DISCUSSION

Method optimization

Many combinations of mobile phase compositions and parameters were tested to optimize the RP-HPLC process. Using a Symmetry C18 HPLC Column (150 mm x 4.6 mm ID, 5 µm) and a mobile phase consisting of 0.1M potassium dihydrogen phosphate buffer (pH adjusted to 6.2 with 0.1% orthophosphoric acid and HPLC grade acetonitrile in the proportion of 55:45, v/v) delivered at a flow rate of 1 mL/min to get better reproducibility and repeatability with wavelength at 246 nm, Column temperature was kept at 25°C, and Runtime was 12 minutes. Ambroxol and Loratadine showed a satisfactory separation and good peak symmetry. The retention times for ambroxol and loratadine were, respectively, 5.894 and 3.202 minutes. Figure 3 displays a typical chromatogram of the standard and sample solution of Loratadine and Ambroxol.

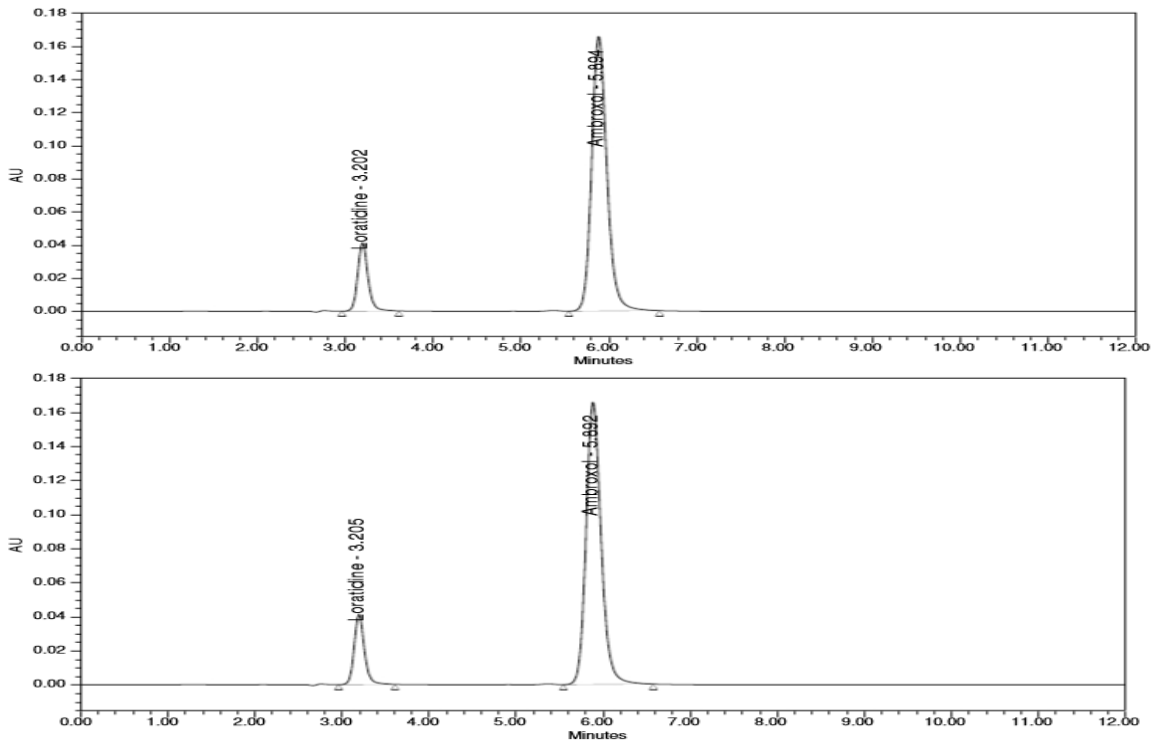


Fig. 3. Chromatogram of standard and sample solution of Loratadine and Ambroxol.

Method validation

The established RP-HPLC method was validated in compliance with ICH guideline Q2 (R1) for the linearity, accuracy, precision, robustness, specificity, reproducibility, sensitivity to obtain the correctness of the method for efficient quality control testing in the laboratory [21-31].

Performance calculations

The system suitability parameters of the suggested RP-HPLC technique for the simultaneous measurement of ambroxol and loratadine in bulk and tablet dosage form were calculated to perform performance calculations. The system suitability of the suggested RP-HPLC method, which is displayed in Table 2, is confirmed by the excellent results of the system suitability parameters for the simultaneous estimation of ambroxol and loratadine in bulk and tablet dosage form. All results fall within the acceptance limit.

Table 2. Performance calculations and system suitability parameters of Ambroxol and Loratadine

Parameters	Ambroxol	Loratadine	Acceptance limits
Retention time (min)	5.894	3.202	-----
Theoretical plates (N)	5812	3722	Not less than 2000
Asymmetry factor	1.1	1.11	Not more than 2
Resolution	10	More than 2	
Linearity range (µg/mL)	15-90	1.25-7.5	-----
Limit of detection (LOD) (µg/mL)	0.03	0.7	-----
Limit of quantification (LOQ) (µg/mL)	0.09	2.18	-----

Specificity

The study examined the impact of excipients and other additives often found in the combined dosage form of ambroxol and loratadine in optimal conditions and verified the absence of any interference.

Linearity

The concentration of 15, 30, 45, 60, 75, and 90 µg/ml of Ambroxol and 1.25, 2.5, 3.75, 5, 6.5, and 7.5µg/ml of Loratadine was prepared for linearity respectively. A 20 µL of each of the aforementioned solutions was injected three times into the HPLC apparatus. Analysis using least squares regression was done to determine the correlation coefficient, slope, and intercept. The calibration curve was constructed by plotting peak area versus concentration (µg/ml) is shown in Figure 4 and the regression equation was calculated and the results are presented in Tables 3 and 4. The correlation coefficient was 0.999 which is considered satisfactory.

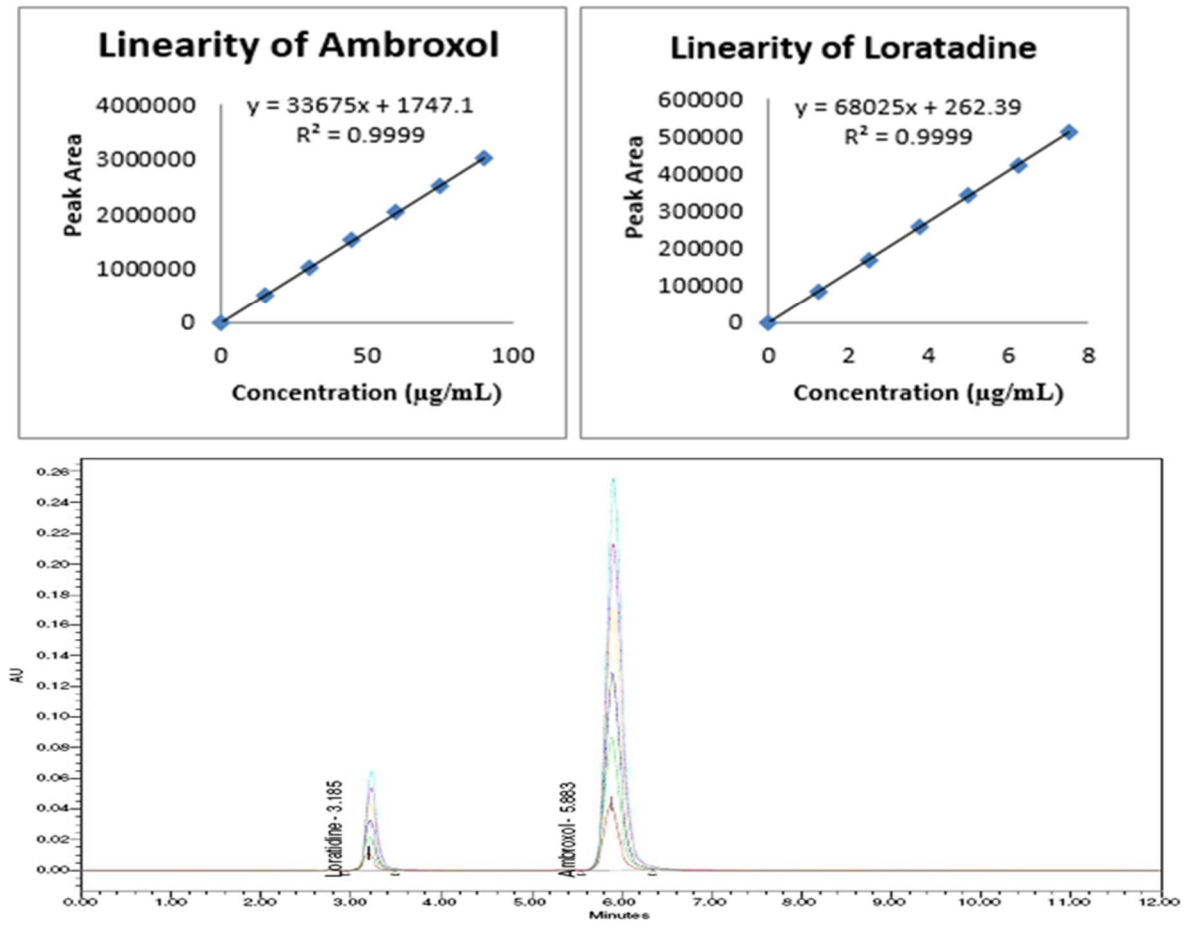


Fig. 4. Standard calibration curves and overlay chromatogram of Ambroxol and Loratadine.

Table 3. Linearity of Ambroxol and Loratadine

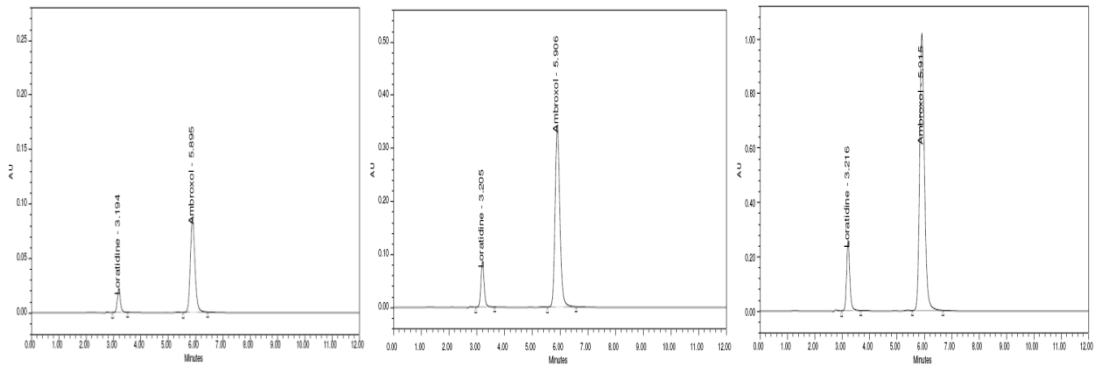
Concentration of Ambroxol (µg/mL)	Peak Area	Concentration of Loratadine (µg/mL)	Peak Area
15	499220	1.25	84860
30	1011151	2.5	170342
45	1521175	3.75	256544
60	2047944	5	342364
75	2515843	6.25	421586
90	3024459	7.5	511803

Table 4. Optical and regression parameters of Ambroxol and Loratadine

Optical and regression parameters	Ambroxol	Loratadine
Detection wavelength (nm)	246	
Linearity range (µg/mL)	15-90	1.25-7.5
Regression Equation (y=mx+C)	33675x+1747	68025x+262.3
Slope (m)	33675	68025
Intercept (C)	1747	262.3

Accuracy

By using the usual addition method to calculate the recoveries of ambroxol and loratadine, the accuracy of the suggested method was calculated. The accuracy of the suggested method was reported in Figure 5 and Tables 5 and 6. The per-analyzed sample solution of Lorfast-AM® tablet powder was mixed with concentration levels of 50%, 100%, and 150% of the standard solution of ambroxol and loratadine to conduct recovery studies.



50% Accuracy **100% Accuracy** **150% Accuracy**

Fig. 5. Accuracy chromatogram of Ambroxol and Loratadine

Table 5. Results of accuracy studies of Ambroxol

Added level	Quantity Added	Quantity Determined	% Recovery	% Mean
50%	30	29.725	99.08	
50%	30	29.868	99.56	99.41
50%	30	29.877	99.59	
100%	60	59.677	99.46	
100%	60	59.622	99.37	99.42
100%	60	59.656	99.43	
150%	90	89.537	99.49	
150%	90	89.804	99.78	99.40
150%	90	89.047	98.94	

Table 6. Results of accuracy studies of Loratadine

Added level	Quantity Added	Quantity Determined	% Recovery	% Mean
50%	2.5	2.485	99.40	
50%	2.5	2.475	99.01	99.14
50%	2.5	2.475	99.01	
100%	5	4.943	98.86	
100%	5	4.967	99.34	99.26
100%	5	4.979	99.57	
150%	7.5	7.437	99.16	
150%	7.5	7.433	99.11	99.11
150%	7.5	7.429	99.06	

Precision

Six injections of homogeneous sample preparation were made into the HPLC system, comprising 60µg/mL of ambroxol and 5µg/mL of loratadine from a single batch sample solution. The results of the method precision of Ambroxol and Loratadine were reported in Table 7.

Table 7. Precision of Ambroxol and Loratadine

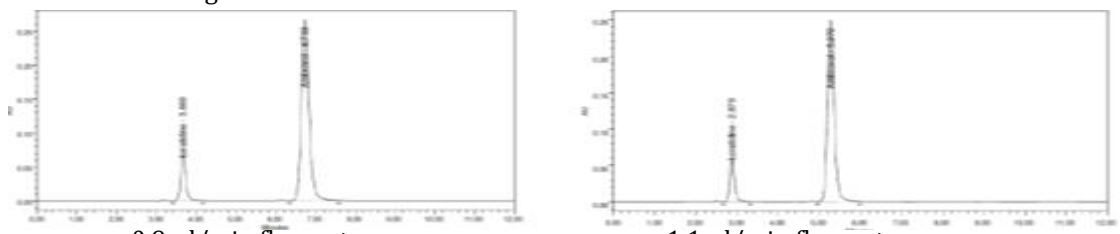
Ambroxol		Loratadine	
Concentration (µg/mL)	Peak Area	Concentration (µg/mL)	Peak Area
60	1992625	5	336142
60	1995024	5	335422
60	1981748	5	332875
60	1994655	5	334881
60	1982458	5	335687
60	1996541	5	332488
Average	1990509	Average	334583
SD	6633.41	SD	1532.86
RSD %	0.33	RSD %	0.46

Limit of detection and Limit of quantitation

The LOD & LOQ were calculated for AMB&LORA. The limit of detection was found to be 0.03µg/ml for AMB and 0.7 µg/ml for LORA respectively. The limit of quantification was found to be 0.09 µg/ml for AMB and 2.18 µg/ml for LORA respectively. The LOD & LOQ values are shown in Table 2 respectively.

Robustness

There were no significant changes which were observed in the developed stability indicating method when the chromatographic conditions viz., Change in the flow rate & Composition of mobile phase were calculated for six injections of AMB & LORA samples. The robustness values and the % RSD results are mentioned in Table 8 and fig 6 and 7.



0.9ml/min flow rate 1.1ml/min flow rate

Fig. 6. Robustness chromatogram for flow rate variations of Ambroxol and Loratadine

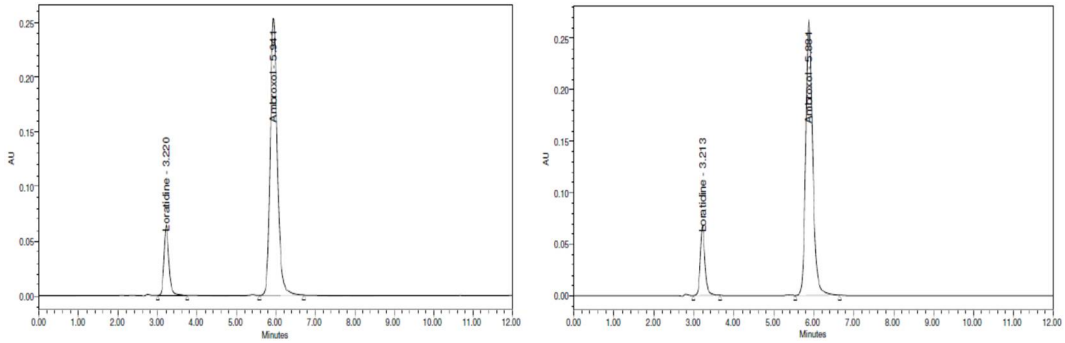


Fig. 7. Robustness chromatogram for mobile phase proportion variations of Ambroxol and Loratadine

Table 8. Robustness data of Ambroxol

Variations in method parameters	Retention Time (mins)	Average peak area	System suitability parameters	
			Theoretical Plates	Asymmetry
Flow rate (0.9ml/min)	6.733	3586391	6065	1.21
Flow rate (1.1ml/min)	5.272	2807773	5276	1.18
-10%, v/v mobile phase	5.941	3188691	5410	1.20
+10%, v/v mobile phase	5.881	3209747	5838	1.19

Table 9. Robustness data of Loratadine

Variations in method parameters	Retention Time (mins)	Average peak area	System suitability parameters	
			Theoretical Plates	Asymmetry
Flow rate (0.9ml/min)	3.660	606040	4184	1.19
Flow rate (1.1ml/min)	2.870	475786	3423	1.18
-10%, v/v mobile phase	3.220	540453	3597	1.19
+10%, v/v mobile phase	3.213	538980	4004	1.16

Forced Degradation Studies

Both Ambroxol and Loratadine were subjected to force degradation conditions viz., acidic, alkaline, neutral, oxidation, photolytic, and thermal. The stress samples of all the degradation were diluted with the prepared diluent to obtain a final concentration of 60 µg/ml and 5µg/ml. The % degradation & % recovery both are calculated and the values are shown in Table 10 & Fig 8-12 respectively.

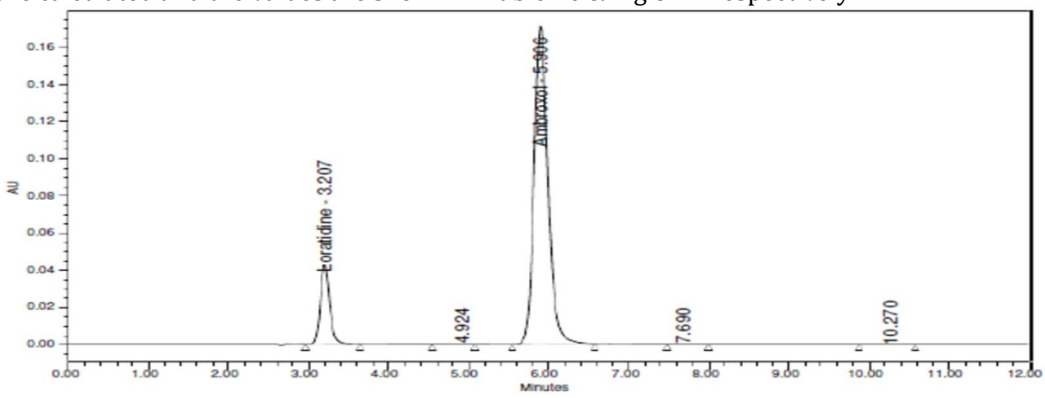


Fig. 8. Chromatogram of acid hydrolysis degradation studies for Ambroxol and Loratadine

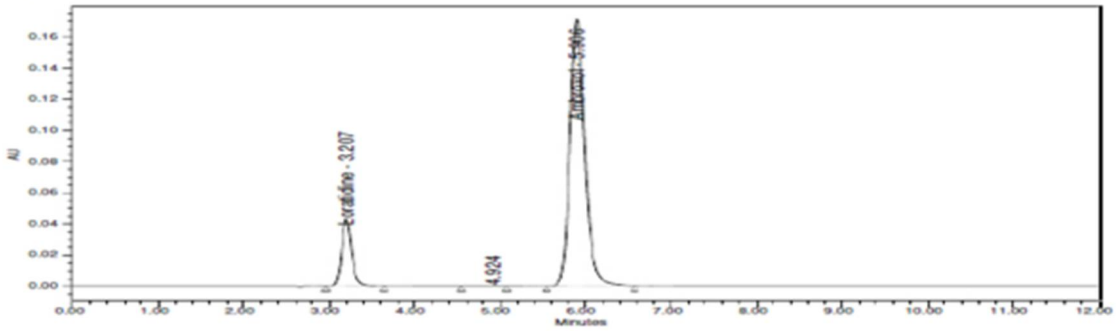


Fig. 9. Chromatogram of alkali hydrolysis degradation studies for Ambroxol and Loratadine

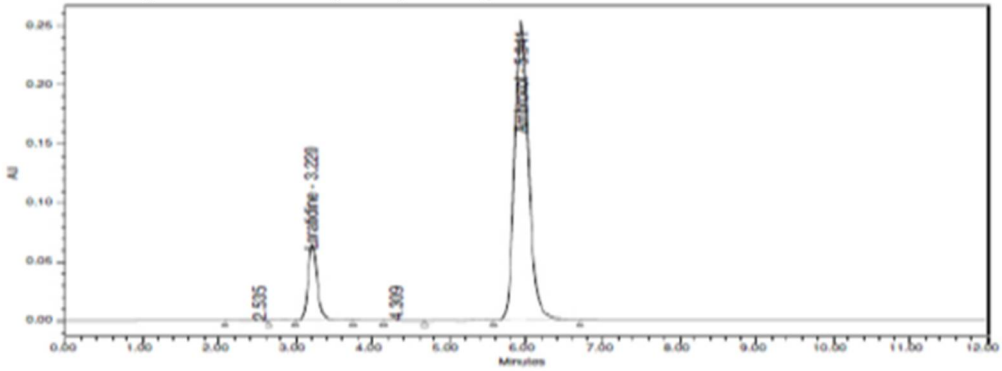


Fig. 10. Chromatogram of oxidative degradation studies for Ambroxol and Loratadine

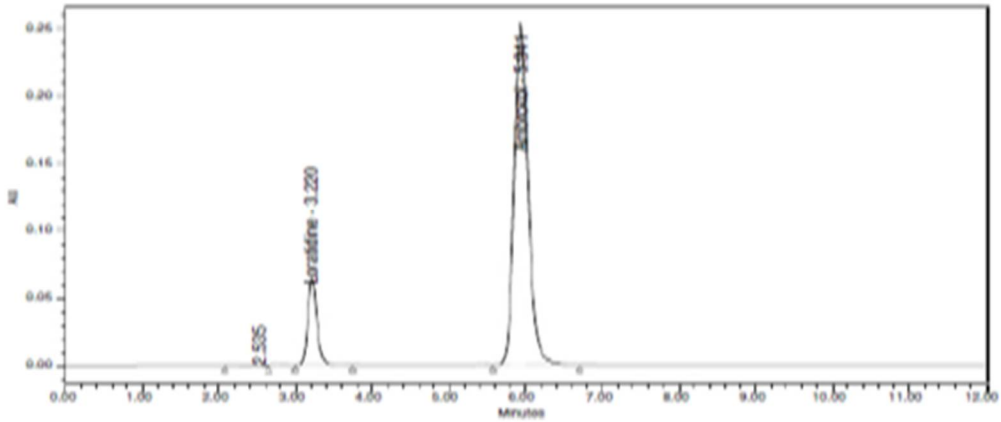


Fig. 11. Chromatogram of thermal degradation studies for Ambroxol and Loratadine

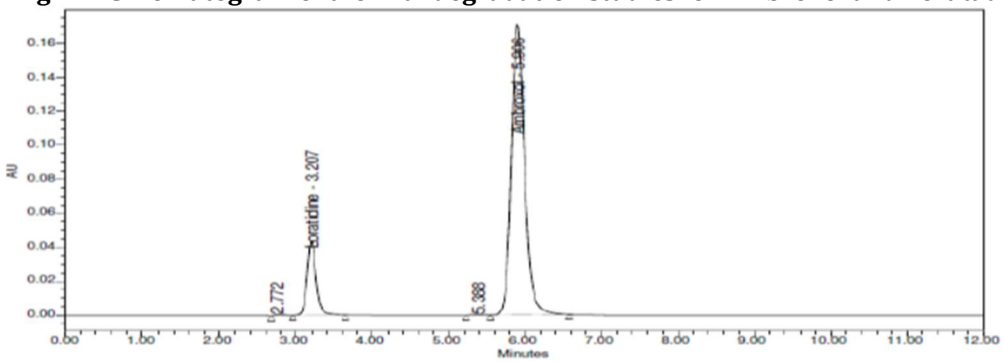


Fig. 12. Chromatogram of photolytic degradation studies for Ambroxol and Loratadine

Table 10. Results of stability indicating assay of Ambroxol and Loratadine

Degradation Condition	Drug Recovered %		Drug Decomposed %	
	Ambroxol	Loratadine	Ambroxol	Loratadine
Acid hydrolysis	96.90	92.87	3.10	7.13
Alkali hydrolysis	95.70	97.10	4.30	2.90
Oxidative degradation	91.50	96.30	8.50	3.70
Thermal degradation	92.88	97.31	7.12	3.69
Photolytic degradation	93.32	96.77	6.68	3.23

Summary of Validation

Table 11- Summary of Validation

Parameters			**AMB**	**LORA**	**Acceptance Criteria**
Specificity	**Retention time**		5.894 min	3.202 min	Specific
Assay	% Assay				98-102%
	% RSD				< 2%
Linearity & Range	Concentration (µg/ml)		15-90 µg/mL	1.25-7.5 µg/mL	Linear
	Correlation coefficient		0.999	0.999	$r^2 \geq 0.998$
Accuracy	50 % (% Recovery)		99.41	99.14	98-102%
	100 % (% Recovery)		99.42	99.26	98-102%
	150 % (% Recovery)		99.40	99.11	98-102%
Precision	(% RSD)		0.33	0.46	< 2%
LOD (µg/ml)			0.03µg/mL	0.70µg/mL	LOD<LOQ
LOQ (µg/ml)			0.09µg/mL	2.18µg/mL	LOQ>LOD
Robustness	Flow rate (ml/min) Rt	0.9	6.733	3.660	Robust
		1.1	5.272	2.870	Robust
	Mobile phase composition Rt	-10	5.941	3.220	Robust
		+10	5.881	3.213	Robust
System suitability	Retention time		5.894	3.202	Resolution was good

	USP Plate count (N)	5812	3722	> 2000
	Asymmetrical factor (ASM)	1.1	1.11	≤ 2.0
	USP Resolution	10		> 1.5

Evaluation of Greenness by AGREE SOFTWARE

The AGREE tool is utilized to assess the environmental sustainability of the existing methodology. AGREE is an analytical tool that may evaluate the environmental impact of analytical methods, regardless of the specific technique utilized. This tool was constructed utilizing the 12 key green analytical chemistry factors, which encompass sample preparation, derivatization, automation, simplicity, solvent toxicity, number of samples tested, number of components analyzed in a single test, energy consumption per analysis, and analysis time. The outcome is transformed into a scale ranging from 0 to 1. The greenness character of the present HPLC technique was assessed using this instrument. The results indicated that the devised approach is environmentally sustainable, achieving a score of 0.72 depicted in Fig no -13.

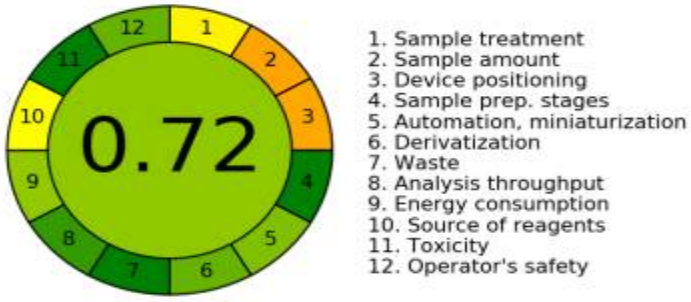


Figure 13 Agree Evaluation

CONCLUSION

Conventional HPLC methods are mostly used in pharmaceutical analysis which have an increased negative impact on the environment. To reduce the impact on the environment, conventional use must be minimized and green methods must be focused. The present study focuses on the development and validation of the RP-HPLC method for simultaneous determination of Amb and Lora along with stability studies in combined dosage form using green assessment tools like AGREE. The developed, optimized, and validated method is economical, environment safe, simple, quick, precise, accurate, robust, etc. This is the first method that has been reported with green analysis of the developed and validated method for estimation of AMB & LORA in a combined form with good resolution, fast elution time, and good peak resolution. The present developed method can be easily applied for quality control tests and routine analysis of AMB & LORA in combined pharmaceutical formulations.

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CITATION OF THIS ARTICLE

S. Swarnalatha, Narmada V, Swetha G, Kavitha P, P. Muralidhar Reddy. Development and Validation of a Stability Indicating Green Analytical Method for The Simultaneous Estimation of Ambroxol and Loratadine in Bulk and Pharmaceutical Dosage forms by RP-HPLC.Bull. Env.Pharmacol. Life Sci., Vol 13 [1] December 2023: 129-140.