IJBPAS, April, 2025, 14(4): 1879-1890

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE IN THEIR COMBINED PHARMACEUTICAL DOSAGE FORMS

PATEL K[1], METRI S[2] AND DAVE H[*1]

1: Department of Pharmaceutical Quality Assurance, Parul Institute of Pharmacy, Parul University, P.O Limda, Tal: Waghodiya, Vadodara PIN Code-391760 Gujarat, India

2: Department of Pharmacognosy, Gokaraju Rangaraju College of Pharmacy, Osmania University, Bachupally PIN – 500049 Hyderabad, India

***Corresponding Author: Dr. Hiral Dave: E Mail: hiral.dave16194@paruluniveristy.ac.in**

Received 16th Nov. 2023; Revised 18th Dec. 2023; Accepted 26th May 2024; Available online 1st April 2025

https://doi.org/10.31032/IJBPAS/2025/14.4.8774

ABSTRACT

The current research aims to develop and validate a stability-indicating reversed phase-high performance liquid chromatographic method for the simultaneous estimation of Doxofylline and Ambroxol Hydrochloride. The specific objective is to create a method that is precise, accurate, and robust, suitable for use as a stability-indicating method for the simultaneous estimation of Doxofylline and Ambroxol Hydrochloride. The method development involved utilizing Chromatopak (250 x 4.6 mm, 5µm) with ACN: Potassium dihydrogen orthophosphate buffer pH 4.5 (pH adjusted with 1 percentage TEA) (70:30) as a mobile phase, detected by a photodiode array detector at a wavelength of 254 nm at a flow rate of 1 mL/min. In this study, Doxofylline and Ambroxol Hydrochloride were exposed to various stress conditions including acidic hydrolysis, basic hydrolysis, neutral hydrolysis, oxidation, thermal, and photolytic degradation. The percentage degradation was found to be within the range of 10-20 percentage. Linearity was observed in the range of 40-200 µg/mL and 3-15 µg/mL with correlation coefficients of 0.998 and 0.999 for Doxofylline and Ambroxol Hydrochloride, respectively. The percentage relative standard deviation of respective observations was found to be less than

2 percentage. The developed method was successfully validated according to ICH guidelines for all parameters for analytical method validation.

Keywords: Ambroxol Hydrochloride, Analytical Method Validation, Asthma, Chronic Obstructive Pulmonary Disease, Doxofylline, Stability indicating RP-HPLC method

INTRODUCTION

Chronic pulmonary disease and asthma are prevalent respiratory diseases that cause difficulty in breathing due to inflammation in the airways **[1]**. Doxofylline (DOX) is an oral methyl xanthine derivative and a phosphodiesterase-4 inhibitor primarily used to treat asthma and Chronic Obstructive Pulmonary Disease (COPD). Its IPUAC name is 7-[(1,3-dioxolan-2-yl) methyl]-1,3 dimethyl-2,3,6,7-tetrahydro-1H-purine 2,6-dione **[2]**. Ambroxol hydrochloride (ABH) is a mucolytic agent that inhibits the non-dependent soluble guanylate cyclase, reducing the overproduction of mucus. This helps maintain low phlegm viscosity and enhances the transfer of bronchial secretions through the mucociliary system. The IUPAC name of ABH is 4-[(2-amino-3,5-dibromophenyl) methylamino] cyclohexan-1-ol. It is commonly used to treat COPD symptoms **[3]**. The structures of DOX and ABH are depicted in **Figure 1 and Figure 2**, respectively.

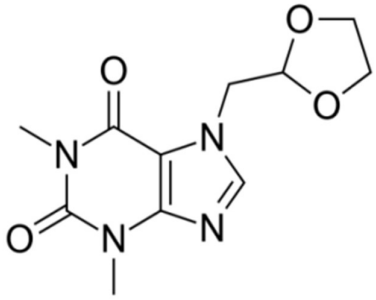

Figure 1: Structure of Doxycycline

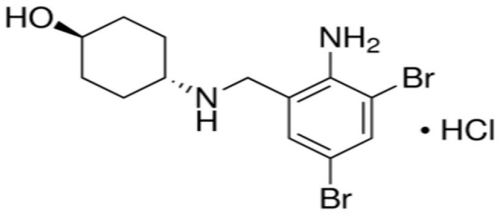

Figure 2: Structure of Ambroxol HCl

The literature provides evidence of various analytical methods for analyzing DOX individually or in combination **[4-9]**, as well as multiple methods for estimating ABH, either alone or in combination with other drugs **[10-12]**. Stability-indicating HPTLC

methods have also been described for simultaneously estimating DOX and ABH in combined dosage forms **[13]**. Looking at the wide applicability and the versatility of reverse-phase HPLC methods, this research article concentrates on the successful development and validation of a stability-indicating reverse-phase HPLC method for both pure Doxofylline and Ambroxol HCl, as well as commercially available formulations in their combined dosage form, along with conducting force degradation studies.

MATERIALS AND METHODS

Chemicals and Reagents

Doxofylline and Ambroxol HCl were received as gift samples from Bakul Finechem Research Centre, Ankleshwar, Gujarat, India, and Akhil Healthcare PVT. LTD., Vadodara, Gujarat, India, respectively. Other common reagents such as potassium dihydrogen phosphate, sodium hydroxide, acetonitrile, triethylamine, hydrochloric acid, and hydrogen peroxide (30%) were of HPLC/AR grade and were obtained from local chemical suppliers.

Instruments

Shimadzu HPLC system model LC-2050C 3D (2.0) with PDA detector was used during the overall process of method development and validation. Apart from this, variety of equipments such as UV Visible Spectrophotometer (Shimadzu 1900), Digital Weighing Balance (Mettler Toledo, Switzerland), FTIR (Bruker, Germany), Melting Point Apparatus (Veego, India) and pH Meter (Elico LIT 20) were used as per the requirement throughout the overall method development and validation.

Preparation of Standard Stock solution

The standard stock solution of Doxofylline and Ambroxol HCl was prepared by accurately weighing 40mg of Doxofylline and 3mg of Ambroxol HCl, respectively. Further dilutions were made for the respective solutions to obtain a total concentration of 4000 µg/mL for DOX and 300 µg/mL for ABH. These solutions were then used to determine the linearity of both drugs after the aid of respective dilutions as per the requirements.

Preparation of mobile phase

The mobile phase was composed of a mixture of acetonitrile and potassium dihydrogen orthophosphate buffer with a pH of 4.5 in a ratio of approximately 70:30. The pH was adjusted using 1% TEA.

Chromatographic conditions

The RP-HPLC analysis was conducted using an HPLC system equipped with a PDA detector, with the UV detection wavelength set at 254 nm. Chromatographic separation was performed on a Chromatopak column (250 ×4.6mm, 5µm). The mobile phase, comprising acetonitrile and potassium dihydrogen orthophosphate buffer at pH 4.5 (adjusted with 1% TEA) in a ratio of 70:30, was delivered at a flow rate

of 1 mL/min. An injection volume of 20 µL was employed, and the total analysis time for each run was 10 minutes.

Procedure for validation of the developed method

System suitability

System suitability was established by injecting all the mixed standards six times. The developed method was validated according to ICH guidelines for parameters, including system suitability, specificity, linearity, range, accuracy, precision, and robustness. An aliquot of the standard solution was mixed and diluted with the mobile phase to achieve a final concentration of 40µg/mL DOX and 3µg/mL ABH. The solution was then injected six times, and system suitability parameters were calculated.

Linearity and Range

From the stock solutions, various dilutions/working standard solutions were prepared for individual drugs in the range of 40-200 µg/mL for DOX and 3-15 µg/mL for ABH, respectively. All the solutions were sonicated for 2 minutes and filtered through a 0.45µm filter paper.

Precision

Precision of the method was determined by repeatability, intraday precision and interday precision.

Repeatability was determined by repeatedly injecting (n=6) a standard solution of 40µg/mL DOX and 3µg/mL ABH into the chromatographic system. The peak areas were measured, and the % relative standard deviation (RSD) was calculated.

Intraday precision was assessed by analyzing three replicates of three concentrations (80, 120, and 180 µg/mL) of the standard solution of DOX and (6, 9, and 12 µg/mL) of ABH on the same day. % RSD was calculated.

At three consecutive days, three concentrations (80, 120, and 180 µg/mL) of DOX and (6, 9, and 12 µg/mL) of ABH were analyzed in triplicate. % RSD was calculated.

Accuracy

The recovery of DOX and ABH was calculated and the accuracy method was determined using the standard addition method. Concentrations within the linearity range were selected, and standards were spiked at three different levels (50%, 100%, and 150%). The amounts of DOX and ABH used to obtain the area value were determined using the regression equation of the calibration.

Robustness

The robustness of the analytical method is determined by its capacity to maintain normal operation even when subjected to small intentional modifications. To confirm robustness, changes were made in the flow rate (±0.2mL/min) and wavelength (±2nm) in the optimised chromatographic

conditions. The percentage % RSD was calculated.

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

The limit of detection (LOD) was estimated from the set of five calibration curves used to determine the method's linearity, and further calculations were performed simultaneously for both drugs using the following equation.

LOD = 3.3 × (SD/Slope)

Where, SD = Standard deviation of the Y-intercepts of the five calibration curves

Slope = Mean slope of the five calibration curves.

In the same way, LOQ was estimated using following equation.

LOQ = 10 × (SD/Slope)

Where, SD = Standard deviation of the Y-intercepts of the five calibration curves.

Slope = Mean slope of the five calibration curves.

Procedure for forced degradation studies

Stress degradation studies were conducted in compliance with ICH guidelines, and investigations were performed using both API and formulations containing ABH and DOX. The drug samples were subjected to forced degradation conditions, including acidic (0.1 HCl for 60 minutes), basic (0.1 NaOH for 60 minutes), peroxide (3% v/v H_2O_2 at room temperature for 60 minutes), neutral (water for 60 minutes), thermal (at 80°C for 60 minutes), and photolytic (near UV fluorescent lamp for 60 minutes) conditions.

Acid degradation

40 mg of DOX and 3 mg of ABH were accurately weighed into separate volumetric flasks. 5 mL of ACN was added to dissolve the substances, followed by 5 mL of 0.1 HCl, and allowed to stand for 60 minutes. Afterward, 1 mL was withdrawn and neutralized by adding up to 1 mL of 0.1 N NaOH. The solution was then diluted with ACN to achieve concentrations of 40 µg/mL for DOX and 3 µg/mL for ABH, respectively.

Base-degradation

40 mg of DOX and 3 mg of ABH were precisely weighed into separate volumetric flasks. Each substance was dissolved in 5 mL of ACN, followed by the addition of 5 mL of 0.1 NaOH, and allowed to stand for 60 minutes. Subsequently, 1 mL was withdrawn, and 1 mL of 0.1 N HCl was added to neutralize it. The resulting solution was then diluted with ACN to achieve concentrations of 40 µg/mL for DOX and 3 µg/mL for ABH, respectively.

Neutral degradation

40 mg of DOX and 3 mg of ABH were accurately weighed into separate volumetric flasks. 5 mL of ACN was added to dissolve the substances, followed by 5 mL of water, and allowed to stand for 60 minutes. Afterward, 1 mL was withdrawn, and 1 mL of neutralized solution was added. The

solution was then diluted with ACN to achieve concentrations of 40 µg/mL for DOX and 3 µg/mL for ABH, respectively

Oxidative degradation

40 mg of DOX and 3 mg of ABH were accurately weighed into separate volumetric flasks. 10 mL of ACN was added to dissolve the substances, followed by 5 mL of 3% H_2O_2, and allowed to stand for 60 minutes. Afterward, 1 mL was withdrawn and diluted with ACN. 1 mL of the above solution was further diluted up to 10 mL, resulting in concentrations of 40 µg/mL for DOX and 3 µg/mL for ABH, respectively.

Thermal degradation

400 mg of DOX and 30 mg of ABH were accurately weighed. The plates were placed in a hot air oven at 80ºC. Subsequently, 40 mg of DOX and 3 mg of ABH were accurately weighed, and 10 mL of diluent was added. 0.1 mL was withdrawn and made up to 10 mL with diluent. This resulted in a solution containing 40 µg/mL of DOX and 3 µg/mL of ABH.

Photolytic degradation

400 mg of DOX and 30 mg of ABH were accurately weighed. The plates were placed in a UV chamber for 1 hour. Subsequently, 40 mg of DOX and 3 mg of ABH were accurately weighed, and 10 mL of diluent was added. 0.1 mL was withdrawn and made up to 10 mL with diluent. This resulted in a solution containing 40 µg/mL of DOX and 3 µg/mL of ABH.

Application of the developed method for commercial formulation analysis

Twenty tablets were accurately weighed and powdered. The powder was dissolved in a sufficient quantity of mobile phase to achieve the final concentration of ABH and DOX within the previously mentioned range. Furthermore, the developed method was applied to a commercial formulation containing ABH and DOX, and the results were recorded, with the core aspects of specificity and simultaneous estimation of ABH and DOX without the aid of prior separation of both the APIs.

The specificity of the HPLC method was also assessed to ensure that there was no interference from excipients and degradants during the simultaneous determination of DOX and ABH. For specificity testing, standard solutions were mixed and diluted with the mobile phase to achieve final concentrations of 40µg/mL for DOX and 3µg/mL for ABH, respectively. From the optimized chromatogram of DOX and ABH, it was observed that there was no interference from any other degradant peak, nor from the excipients of the formulation.

RESULTS AND DISCUSSION

The method was optimized following a detailed study of the structure of both drugs and their primary parameters. After numerous trials, a method utilizing Chromatopak C18 (250 mm × 4.6 mm id, 5 µm particle size) with a mobile phase ratio

of Acetonitrile: potassium dihydrogen orthophosphate buffer- (70:30) of pH 4.5 (pH adjusted with 1% TEA), was selected as one of the key optimised parameter. The flow rate of the mobile phase was optimised at 1 mL/min and detection wavelength was optimised at 254nm for overall method development and validation. Validation parameters and forced degradation studies were conducted, and the chromatogram and results were subsequently calculated. The solution was injected six times, and system suitability parameters were calculated. A representative chromatogram of system suitability was depicted in **Figure 3**.

In accordance with the methodology, accuracy was evaluated by determining the recovery of the standards of DOX and ABH respectively. The results of the recovery were presented in **Table 1**.

As stated in the methodology, the robustness of the method was assessed by making minor modifications to the optimized method parameters. Following the same approach, the results of the robustness testing were documented in the **Table 2** provided below.

The developed method was successfully validated as per ICH guidlines, and the results of all validation parameters were presented in **Table 3**.

Results of forced degradation studies

Forced degradation studies entailed subjecting drug substances and products to conditions more severe than those encountered in accelerated situations. In the presence of diverse stress conditions such as acidic, basic, neutral environments, elevated temperatures, and exposure to light, the results revealed effective differentiation between the peaks of the pure drugs, DOX and ABH, in terms of their respective areas under the curves at standard retention times, even in the presence of degradant peaks.

Following the application of various stress conditions, the corresponding chromatograms were depicted in the subsequent figures. Specifically, **Figure 4a** illustrates the chromatogram of acid degradation, **Figure 4b** reveals the chromatogram of basic degradation, and **Figure 4c** represents the chromatogram of neutral degradation.

Similarly, **Figure 4d, Figure 4e, and Figure 4f** depict the chromatograms for oxidative, thermal, and photolytic degradation, respectively.

The degradation percentage corresponding to each type of stress condition applied is calculated, and the outcomes are documented in **Table 4**, presented below. Additionally, the results are also summarized in **Table 4**.

Application of the Developed Method for Commercial Formulation Analysis

The developed method was successfully tested and found to be specific, demonstrating no interference of excipients in the simultaneous determination of DOX and ABH. Various commercial brands of DOX and ABH were successfully analysed, and the results are recorded as follows in **Table 5**.

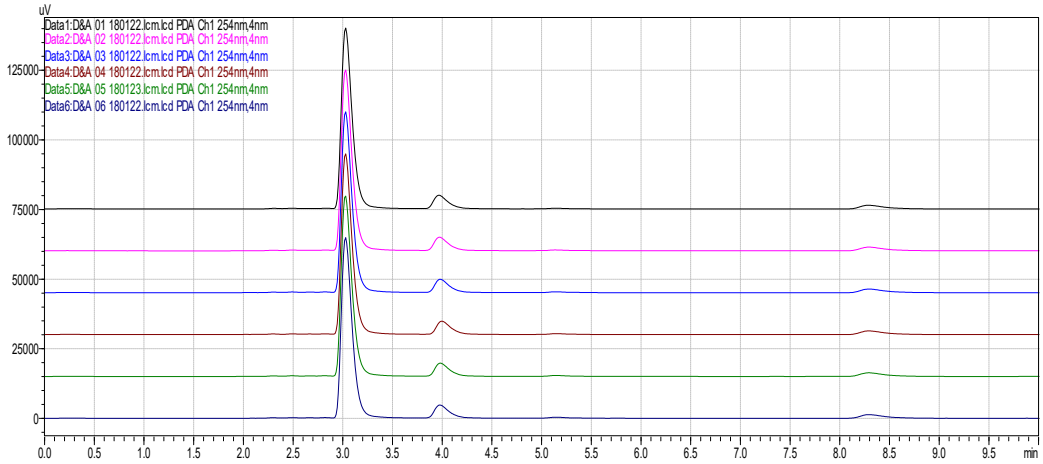

Figure 3: System suitability of DOX and ABH in mixture

Table 1: Results of recovery study for DOX and ABH

Drug	% Level (Spiking)	Amount of sample mixture (µg/ml)	Amount of Standard spiked (µg/ml)	%Recovery*	Mean Recovery	S.D.
DOX	50	106	53	99.7±0.18	99.52	0.253
	100	106	106	99.91±0.29		
	150	106	159	98.97±0.29		
ABH	50	8	4	98.88±0.20	99.21	0.25
	100	8	8	99.17±0.33		
	150	8	12	99.6±0.22		

*Mean value of three determinations; SD: Standard Deviation

Table 2: Results of robustness testing

Parameters	% RSD	
	DOX*	ABH*
Change in detection Wavelength		
1. 252nm	0.1338	0.1574
2. 254nm	0.5900	0.4431
3. 256nm	0.7536	0.4517
Change in Flow rate of mobile phase		
1. 0.9mL/min	0.1262	0.1208
2. 1.0mL/min	1.0974	0.9273
3. 1.1mL/min	0.1434	0.2965

*Mean values of three determinations; %RSD - %Relative Standard Deviation

Table 3: Results of Validation Parameters

Parameters	DOX	ABH
Wavelength (nm)	254nm	
Range	40-200 µg/mL	3-15 µg/mL
Slope	10049	12135
Intercept	28125	7884
Correlation coefficient	0.9993	0.9986
Accuracy	99.52 ± 0.253	99.21 ± 0.25
Intraday Precision*(%RSD)	0.439	0.224
Inter day Precision*(%RSD)	1.241	0.956
LOD (µg/ml)	3.457	0.562
LOQ (µg/ml)	10.477	1.705
Robustness Different wavelength (%RSD) Different flow rates (%RSD)	 0.492 0.455	 0.35 0.448
Repeatability** (%RSD)	0.411	0.310
System suitability Parameters** Theoretical Plates (Mean ± SD) Retention time (Mean ± SD) Tailing Factor (Mean ± SD) Resolution (Mean ± SD)	 3201.6 ± 0.177 3.02 ± 0.0008 1.3 ± 0.003 ----------	 3277.3 ± 42.35 3.02 ± 0.0108 1.41 ± 0.005 3.86 ± 0.049

*n= Mean value of three determinations; **n=Mean value of six determinations; SD= Standard Deviation
%RSD=%Relative Standard Deviation

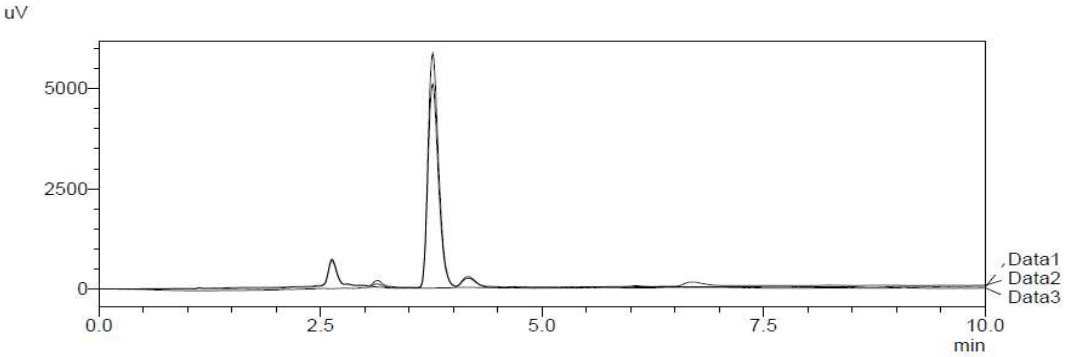

Figure 4a: Overlay chromatogram of test solution for acid degradation at 60 minutes

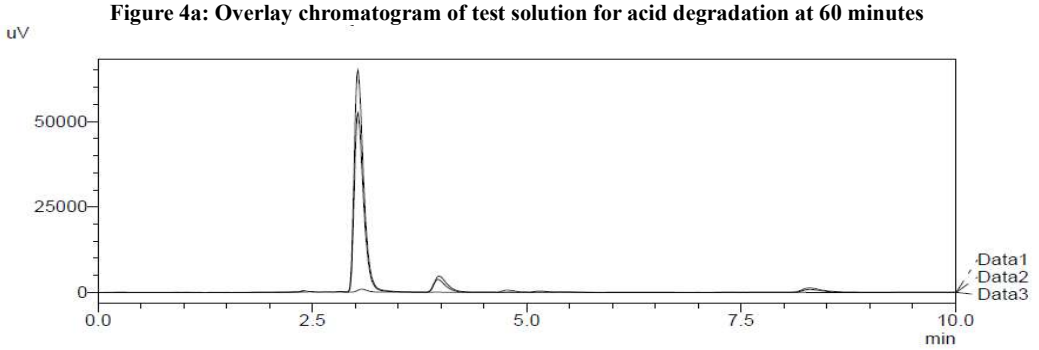

Figure 4b: Overlay chromatogram of test solution for base degradation at 60 minutes

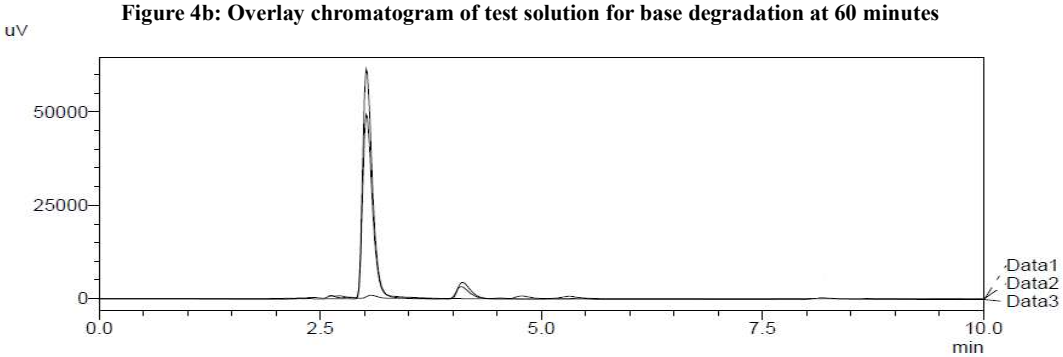

Figure 4c: Overlay chromatogram of test solution for neutral degradation at 60 minutes

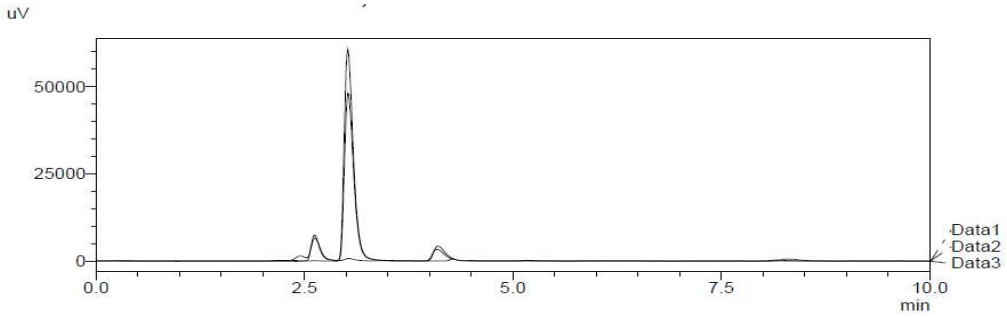

Figure 4d: Overlay chromatogram of test solution for oxidative degradation at 60 minutes

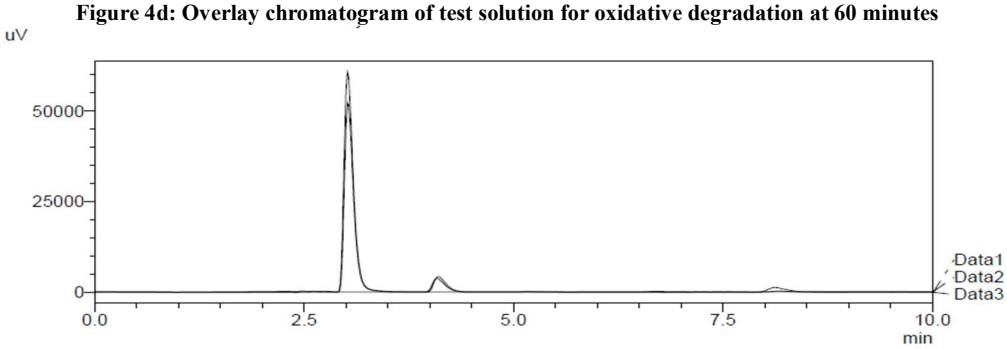

Figure 4e: Overlay chromatogram of test solution for thermal degradation at 60 minutes

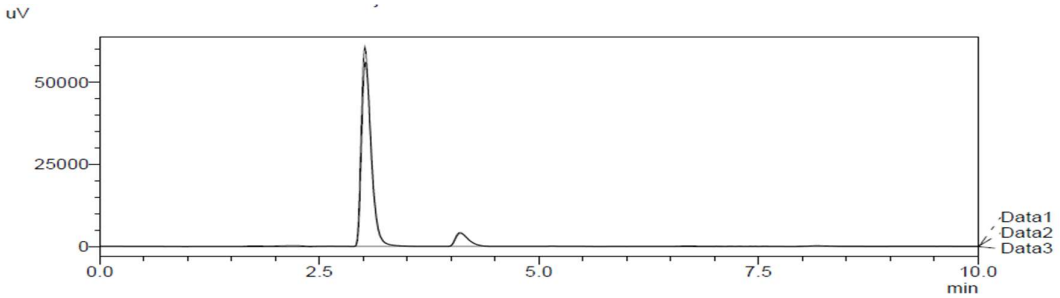

Figure 4f: Overlay chromatogram of test solution for photolytic degradation at 60 minutes

Table 4: Results of the percentage degradation for DOX and ABH after the application of a variety of stress conditions

Sr. No	Stress Condition	% Degradation	
		DOX	ABH
1.	Acidic degradation	19.10	19.00
2.	Basic degradation	19.85	20.31
3.	Neutral degradation	16.57	30.33
4.	Oxidative degradation	17.44	18.14
5.	Thermal degradation	15.78	18.90
6.	Photolytic degradation	16.35	14.15

Table 5: Commercial formulation analysis of DOX and ABH

Brands	Label Claim* (mg/mixture)		Amount Found (mg/mixture)		% Label Claim (mg/mixture)	
	DOX	AMB	DOX	AMB	DOX	AMB
Brand I	400	30	399.35	29.51	99.83	98.36
Brand II	400	30	398.97	30.03	99.74	100.1
Mean	----		-----		99.83	99.39
S.D.					0.100	0.916

*-mean value for three determinations

CONCLUSION

A stability-indicating RP-HPLC method is successfully developed and validated for the simultaneous determination of Doxofylline and Ambroxol HCl in their combined pharmaceutical formulations. The developed method, noted for its simplicity, speed, accuracy, precision, and specificity, facilitates the effective determination of both Doxofylline and Ambroxol HCl concentrations within their combined dosage forms. Furthermore, it serves as a reference for future research endeavors aimed at developing and validating stability-indicating methods for various drug binary mixtures.

REFERENCES:

[1] Postma DS, Reddel HK, ten Hacken NH, van den Berge M. Asthma and chronic obstructive pulmonary disease: similarities and differences. Clinics in chest medicine. 2014 Mar 1;35(1):143-56.

[2] Ren C, Zhao H, Farajtabar A, Zhu P. Quantitative molecular surface analysis of doxofylline and its thermodynamic solubility behavior in aqueous solutions. The Journal of Chemical Thermodynamics. 2022 Aug 1;171:106792.

[3] Manjunatha NK, Kumar SM, Swamy MT, Siddaraju BP, Lokanath NK. Solid state and Computational studies of Ambroxol hydrochloride drug. Chemical Data Collections. 2020 Jun 1;27:100377.

[4] Samanthula G, Yadiki K, Saladi S, Gutala S, Surendranath KV. Stability-indicating RP-HPLC method for the simultaneous estimation of doxofylline and terbutalinesulphate in pharmaceutical formulations. Scientia Pharmaceutica. 2013 Dec;81(4):969-82.

[5] Sakhare RS, Sanjay SP., Development and validation of stability-Indicating high-performance thin-layer chromatography method for the determination of acebrophylline and doxofylline in bulk and combined dosage form. J Pharma Research. 2018;1.

[6] Nirupa G, Siva Kumar A, Tripathi UM. Novel LC Method Development and Validation for Simultaneous Determination of Montelukast and Doxofylline in Bulk and Pharmaceutical Dosage Forms. Journal of Chemistry. 2013 Jan 1;2013.

[7] Patel JJ, Chorawala H, Dedania ZR, Vijendraswamy SM. Development and Validation of

UV Spectroscopic Method for Simultaneous Estimation of Doxofylline and Terbutaline Sulphate in Combined Dosage Form. Asian Journal of Pharmaceutical Analysis. 2015;5(2):74-8.

[8] Samanthula G, Yadiki K, Saladi S, Gutala S, Surendranath KV. Stability-indicating RP-HPLC method for the simultaneous estimation of doxofylline and terbutalinesulphate in pharmaceutical formulations. Scientia Pharmaceutica. 2013 Dec;81(4):969-82.

[9] Sakhare RS, Sanjay SP, Development and validation of stability-Indicating high-performance thin-layer chromatography method for the determination of acebrophylline and doxofylline in bulk and combined dosage form. J Pharma Research. 2018;1.

[10] Thote L, Dhabarde D, Baheti J, Borkar S, Bhusari S. Development and Validation Method for Simultaneous Investigation of Ambroxol Hydrochloride and N-Acetyl Cysteine In Bulk And Pharmaceutical Formulation By RP-HPLC. Asian Journal of Pharmaceutical Research and Development. 2022 Dec 14;10(6):38-43.

[11] Kumar R, Bansal M, Agarwal D. Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Salbutamol Sulphate and Ambroxol Hydrochloride in Oral Liquid Dosage Form. Asian Journal of Pharmaceutical Research and Development. 2021 Feb 13;9(1):84-94.

[12] Patel DK, Vyas AJ, Noolvi M, Patel AB, Patel NK. Estimation of four drugs: Ambroxol hydrochloride, levocetirizine hydrochloride, phenylephrine hydrochloride and paracetamol by RP-HPLC in tablet dosage form. Int. J. Pharmaceut. Chem. Anal. 2018;5(1):8-31.

[13] Patel KG, Shah PS, Gandhi TR. Stability-indicating high-performance thin-layer chromatographic method for the estimation of ambroxol hydrochloride and doxofylline in a pharmaceutical formulation using experimental design in robustness study. JPC-Journal of Planar Chromatography-Modern TLC. 2016 Apr;29(2):132-9.