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STABILITY INDICATING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF ACEBROPHYLLINE AND DOXOFYLLINE IN PHARMACEUTICAL DOSAGE FORM

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Keywords:

Acebrophylline, Doxofylline, RP-HPLC, Stability, Stress degradation.

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ABSTRACT: Objective: To develop a simple, selective, and precise stability-indicating high-performance liquid chromatography method for the simultaneous estimation of acebrophylline and doxofylline in bulk and tablet dosage form. **Methods:** The chromatographic separation achieved on HiQSil C18 Column (250 × 4.6 mm, 5µm) utilizing a mobile phase Acetonitrile: 10 mM n-hexane sulfonic acid buffer (80: 20, v/v) at a flow rate of 1.0 ml/min with injection volume 20 µl. UV detection was performed at 250 nm. The method was validated as per ICH guidelines. **Results:** The retention time for acebrophylline and doxofylline was found to be 2.77 min and 9.56 min, respectively. The linear regression analysis data for the calibration plots showed a good linear relationship in the concentration range of 1-10 µg/ml for acebrophylline and 4-24 µg/ml for doxofylline. The percentage recoveries of acebrophylline and doxofylline in the marketed dosage form were found to be 99.91 and 94.24, respectively. The correlation coefficients for acebrophylline and doxofylline were 0.997 and 0.998, respectively. The percentage degradation at different stress conditions like acid, alkaline, Neutral, oxidative, Dry heat, and photolytic for acebrophylline were found to be 14.84, 10.17, 9.5,11.34, 0.00 and 5.45 respectively and for doxofylline, found to be 8.19, 11.57, 12.74, 8.38, 9.57 and 11.02 respectively. **Conclusion:** The developed method was successfully validated as per ICH guidelines. This method is simple, selective, linear, precise, accurate, and sensitive and can be applied for routine estimation of tablet dosage forms containing both drugs.

INTRODUCTION: Acebrophylline is an anti-inflammatory and airway mucus regulator. It contains ambroxol and theophylline-7-acetic acid. That facilitates the biosynthesis of pulmonary surfactant while later raises blood levels of ambroxol, by stimulating surfactant production [1]. Chemically acebrophylline **Fig. 1A** is (1, 3-dimethyl-2, 6- dioxo-1, 2, 3, 6- tetrahydro-7H-purine-7yl) acetic acid-4 [((2-amino-3, 5-dibromophenyl) methyl) amino] cyclohexanol.

It is a salt obtained by reaction of equimolar amounts of theophylline-7-acetic acid and ambroxol [2]. Theophylline-7-acetate has a bronchodilator effect due to inhibition of the intracellular phosphodiesterases, followed by an

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increase of adenosine mono phosphate cyclic levels, which promote the relaxation of bronchial muscles. Doxofylline (DOX) is xanthine derivative, chemically it is 7-(1, 3-Dioxolan-2-methyl)-3, 7-dihydro- 1, 3-dimethyl-1H-purine-2, 6-dione **Fig. 2B.** It is used in the treatment of asthma. Its mechanism of action is related to the inhibition of Phospho-diesterase activity, leading to increased levels of cyclic nucleotides, thus causing bronchodilation [3].

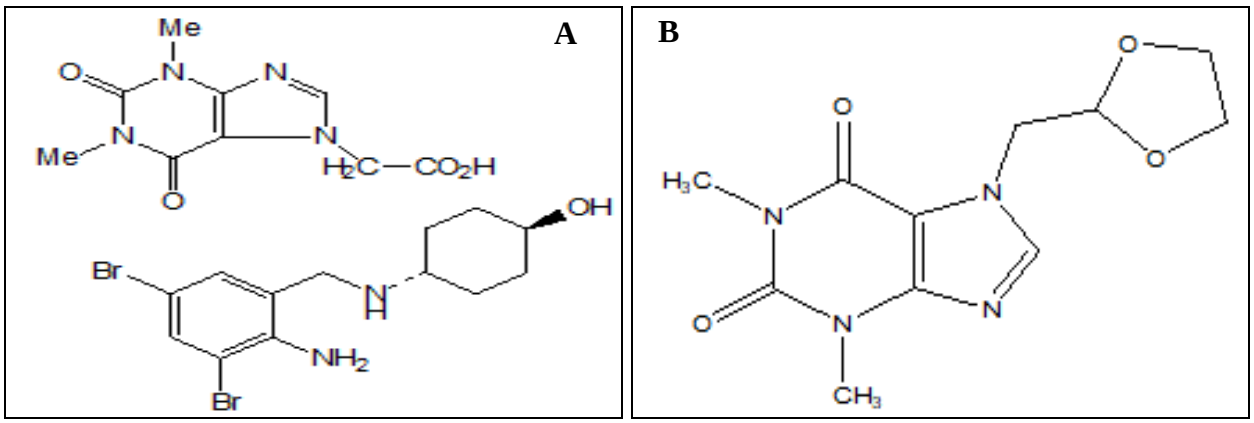

FIG. 1: CHEMICAL STRUCTURE OF ACEBROPHYLLINE (A) AND DOXOFYLLINE (B)

The standard solution was prepared to contain 1 µg/ml of ACEBRO. The standard stock solution of DOXO was prepared by dissolving 40 mg of drug in 10 ml of Acetonitrile to get a 4000 µg/ml concentration. From the standard stock solution, the working standard solution was prepared to contain 4 µg/ml of DOXO.

Preparation of Sample Solution of Combination Tablets: Twenty tablets containing 100 mg of ACEBRO and 400 mg of DOXO were weighed and powdered. Powder equivalent to 10 mg of ACEBRO (40 mg of DOXO) was transferred to 10 ml volumetric flask and was diluted with Acetonitrile. It was sonicated for 10 min and filtered.

Then the volume was made to 10 ml with Acetonitrile to obtain the concentration of 1000 µg/ml for ACEBRO and 4000 µg/ml for DOXO. Further dilutions were made with mobile phase to get the final concentration of 2 µg/ml of ACEBRO and 8 µg/ml of DOXO and were used as a working solution.

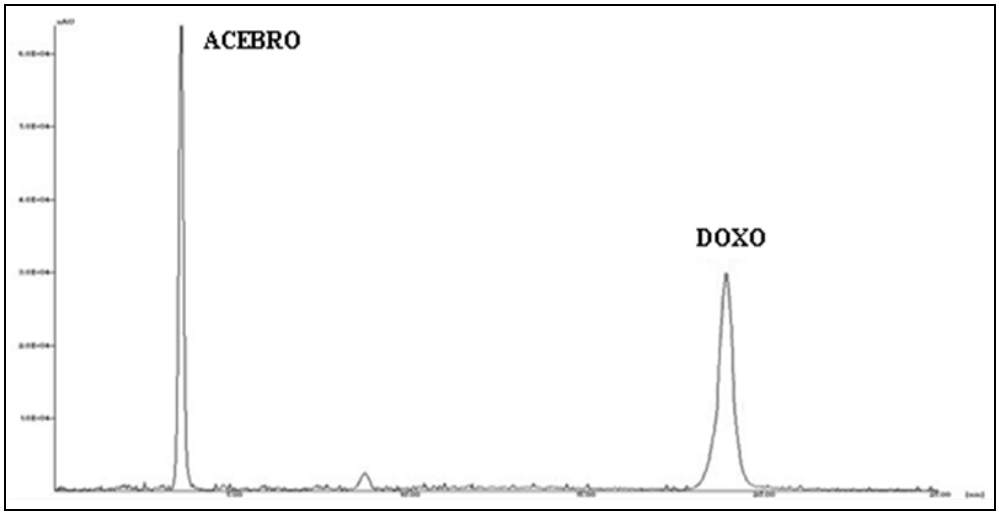

FIG. 2: CHROMATOGRAM OF ACEBRO (10 µG/ML, RT = 2.77 ± 0.04 MIN) AND DOXO (10 µG/ML, RT = 9.56 ± 0.15 MIN)

Method Validation: As recommended in the ICH guidelines, all validation was performed during the development of the procedure. The analytical method was validated for linearity, accuracy, precision, the limit of detection (LOD), Limit of quantification LOQ), Robustness and specificity.

Precision: Repeatability, intra-day, and inter-day precision studies were carried out by estimating corresponding responses three times on the same Arrange Properly,

Remove full stop after one and after of Acebrophyl line (4, 6, 8 µg/ml) and Doxofylline (12, 16, 20 µg/ml) and results are reported in terms of % relative standard deviation.

TABLE 1: PRECISION STUDY DATA OF ACEBRO AND DOXO

Parameter	ACEBRO			DOXO		
	Amount taken(µg/ml)	Amount found (%)	% RSD	Amount taken(µg/ml)	Amount found(%)	% RSD
Intra-day [n= 3]	4	99.41	0.87	12	98.94	0.75
	6	99.85	1.33	16	99.85	0.50
	8	100.58	0.69	20	99.78	0.66
Inter-day [n= 3]	4	99.83	0.76	12	99.13	0.47
	6	99.77	0.78	16	99.39	1.09
	8	100.24	0.57	20	99.35	1.43

TABLE 2: RECOVERY STUDY OF ACEBRO AND DOXO

Drug	Amount taken (µg)	Amount of standard drug added (µg)	Amount Recovered (µg)	% Amount Recovered	% R.S.D.*
ACEBRO	2	1.6	3.59	99.74	0.70
	2	2	4.0034	100.07	1.74
	2	2.4	4.38	99.61	1.01
DOXO	8	6.4	14.36	100.01	0.59
	8	8	15.95	99.71	0.81
	8	9.6	17.44	99.13	1.00

*Average of three determination

Linearity: Linearity was studied by preparing standard solutions at different concentrations from 1-10 µg/ml and 4-24 µg/ml for ACEBRO and DOXO resp. plotting a graph of concentration against peak area and determining the linearity by least-squares regression.

TABLE 3: LINEARITY STUDY DATA OF ACEBRO AND DOXO

Drug Name	Conc.(µg/ml)	Area	Drug Name	Conc.(µg/ml)	Area
ACEBRO	1	109236		4	151352
	2	125614	DOXO	8	277561
	4	155092		12	434327
	6	183491		16	581329
	8	214484		20	746439
	10	236728		24	893327
	Correlation Coefficient (r^2)	0.9979		Correlation Coefficient (r^2)	0.9988
	Regression Equation	y=14317x+96805		Regression Equation	y=37618x-13096

Limit of Detection and Limit of Quantitation: LOD and LOQ for both the drugs were calculated by using following formula as per ICH guidelines.

$$LOD=3.3 \times S.D/S, LOQ=10 \times S.D/S$$

Name of the drug	LOD (µg/ml)	LOQ (µg/ml)
ACEBRO	0.56 µg/ml	1.70 µg/ml
DOXO	0.44 µg/ml	1.33 µg/ml

Where, SD- the standard deviation of the responses and S is the slope of the calibration plot.

Accuracy, as Recovery: Accuracy was evaluated in triplicate, at three different concentrations equivalent to 80,100 and 120% of the active ingredient, by adding a known amount of ACEBRO and DOXO to a sample of known concentration and calculating the recovery, % RSD of ACEBRO and DOXO for each concentration.

Robustness: The robustness of the HPLC method was studied by changing flow rate (± 0.1 ml/min) and working wavelength (± 1 nm).

Specificity: The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than

99.10, indicating no interference of any other peak of a degradation product, impurity or matrix.

System Suitability Test: Analytical system performance before and/or during the analysis was evaluated by a system suitability test. System suitability tests are an integral part of method development. They are performed to evaluate the behavior of the chromatographic system, such as capacity factor (k), plate number (N), Asymmetry factor.

TABLE 4: ROBUSTNESS STUDY DATA

Drug	% RSD found for Robustness study					
	Flow Rate (1ml/min)			Wavelength (249 nm)		
	0.9 min	1.0 min	1.1 min	250 nm	249 nm	251 nm
ACEBRO	0.56	0.39	0.97	0.23	0.71	0.37
DOXO	0.43	0.69	0.76	0.49	0.66	0.65

TABLE 5: SYSTEM SUITABILITY DATA

Name of Drug	RT (Min)	Tailing Factor (T)	Theoretical Plates (N)	Asymmetry Factor
ACEBRO	2.77±0.04	0.89	6604	1.013
DOXO	9.56±0.15	1.24	7235	1.210

Forced Degradation Studies:

Acid and Base Induced Degradation: Acid and Base induced degradation was performed by adding 1 ml of stock solution of ACEBRO and DOXO to 1 ml of 0.1N HCl, and 0.1 N NaOH was kept in a dark place at 300 °C. The resultant solution was diluted to obtain 6 µg/ml and 16 µg/ml solution of ACEBRO and DOXO. Then injected into the system and chromatogram were recorded to assess the stability of sample **Fig. 3-6.**

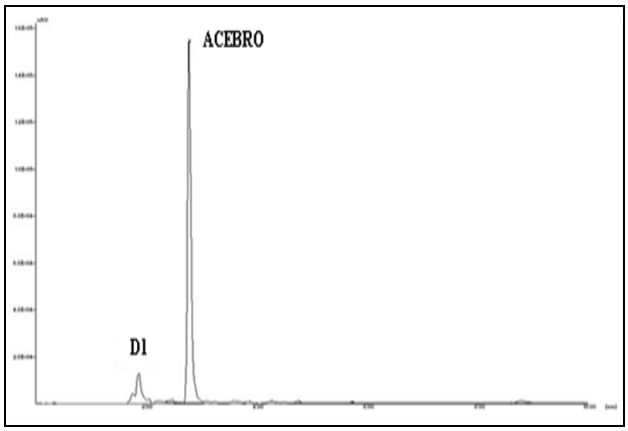

FIG. 3: CHROMATOGRAM OF ACEBRO AFTER ACID DEGRADATION (D1= 1.8 MIN)

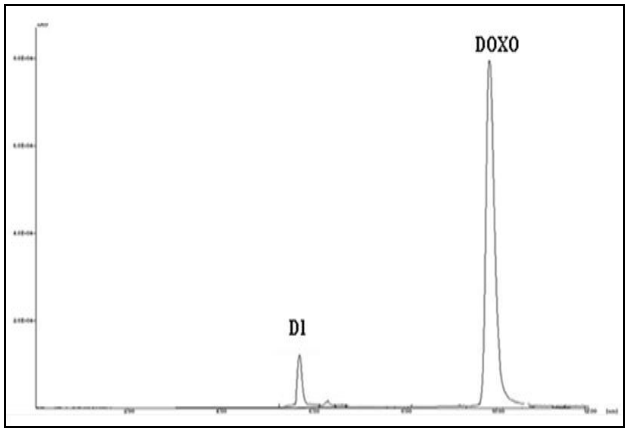

FIG. 4: CHROMATOGRAM OF DOXO AFTER CID DEGRADATION (D1= 5.82 MIN)

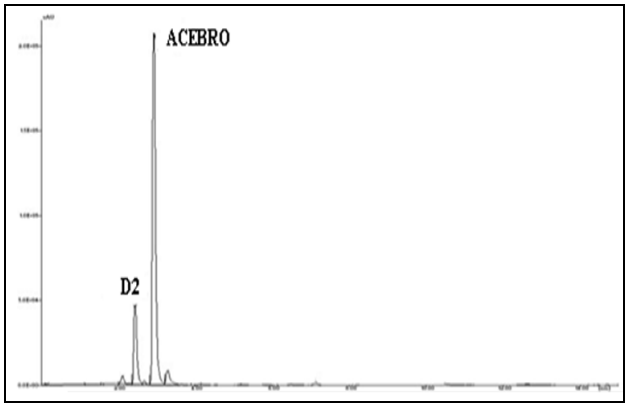

FIG. 5: CHROMATOGRAM OF ACEBRO AFTER ALKALINE HYDROLYSIS (D2= 2.51 MIN)

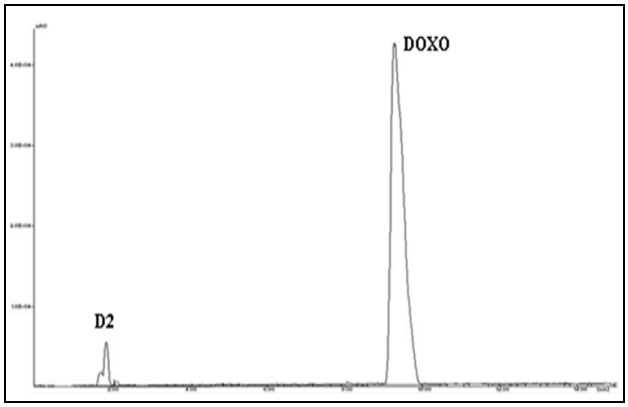

FIG. 6: CHROMATOGRAM OF DOXO AFTER ALKALINE HYDROLYSIS (D2= 1.91 MIN

Neutral Hydrolysis: 1 ml working standard solution of ACEBRO (1000 µg/ml) was mixed with 1 ml water and 8 ml of methanol. The solution was kept for 30 min in a dark place. The 0.6 ml of the resulting solution was diluted with mobile phase up to 10 ml and then was injected (6 µg/ml). For DOXO, 1 ml working standard solution (4000 µg/ml) was mixed with 1 ml water and 8 ml of methanol. The solution was kept for 30 min in a dark place. The 0.4 ml of the resulting solution was diluted with mobile phase up to 10 ml and then was injected 16 µg/ml **Fig. 7** and **8.**

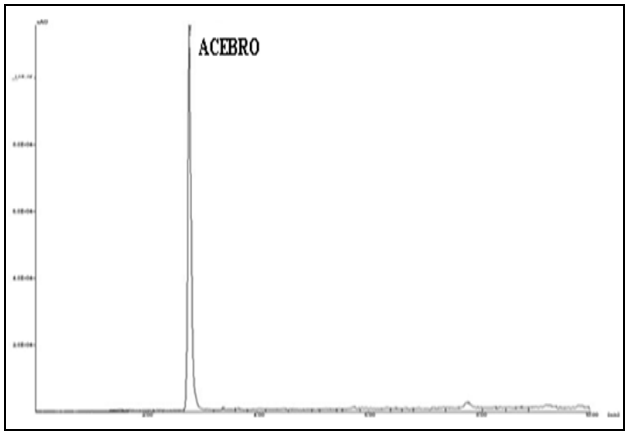

FIG. 7: CHROMATOGRAM OF ACEBRO AFTER NEUTRAL HYDROLYSIS

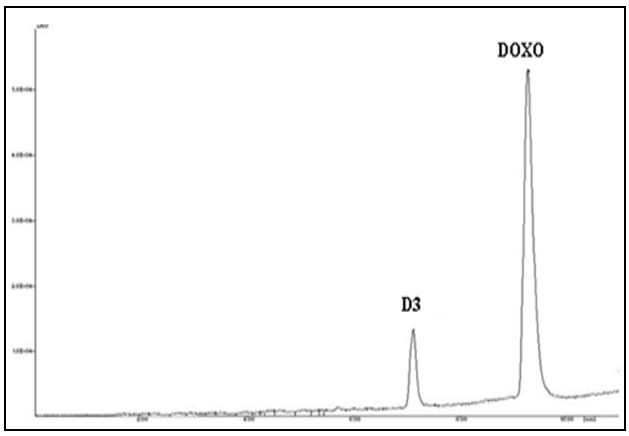

FIG. 8: CHROMATOGRAM OF DOXO AFTER NEUTRAL HYDROLYSIS (D3=7.3 MIN)

Oxidation: Oxidative degradation study was performed by adding 1 ml of stock solution of ACEBRO and DOXO to 1 ml of 30% H_20_2, and the solution was kept for 30 min. in a dark place. The resultant solution was diluted to obtain 6 µg/ml and 16 µg/ml of ACEBRO and DOXO resp. then this solution is injected into the system, and chromatograms were recorded to assess the stability of samples **Fig. 9** and **10.**

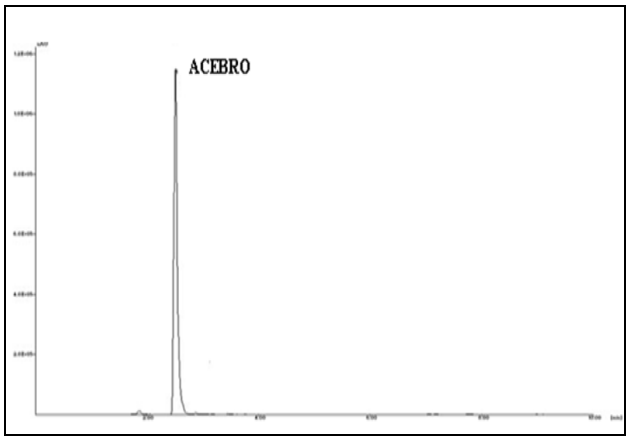

FIG. 9: CHROMATOGRAM OF ACEBRO AFTER OXIDATION

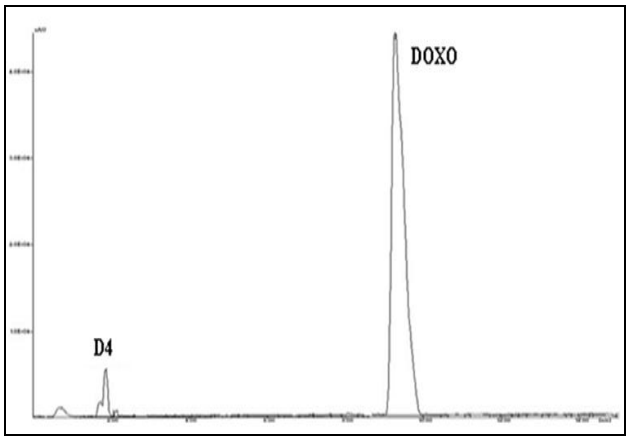

FIG. 10: CHROMATOGRAM OF DOXO AFTER OXIDATION (D4=1.92 MIN)

Degradation Under Dry Heat: Dry heat studies were performed by keeping drug samples ACEBRO and DOXO separately in an oven 1000 °C for a period of 1 h.

Samples were withdrawn after 1 hour, dissolved in acetonitrile to get the solution of 1000 µg/ml for ACEBRO and 4000 µg/ml for DOXO, and these solutions were diluted separately with mobile phase to get 6 µg/ml and 16 µg/ml as final concentration for ACEBRO and DOXO, respectively and were injected **Fig. 11** and **12.**

Photo-degradation Studies: The photochemical stability of the drug was also studied by exposing the stock solution to UV- light for 200 watts/m^2. The resultant solution was diluted to obtain 6 µg/ml and 16 µg/ml as final concentrations for ACEBRO and DOXO, respectively, and injected into the HPLC **Fig. 13** and **14.**

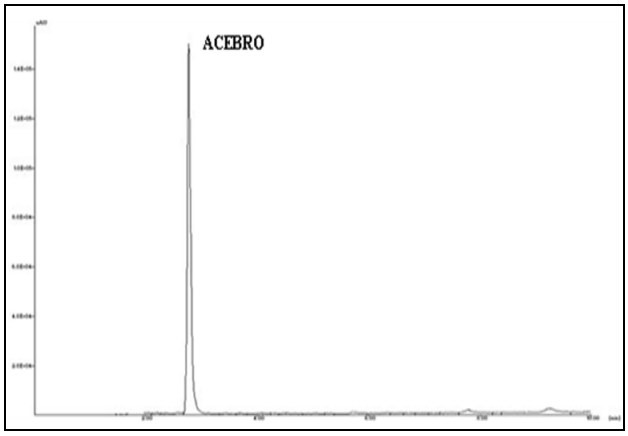

FIG. 11: CHROMATOGRAM OF ACEBRO AFTER EXPOSING TO DRY HEAT

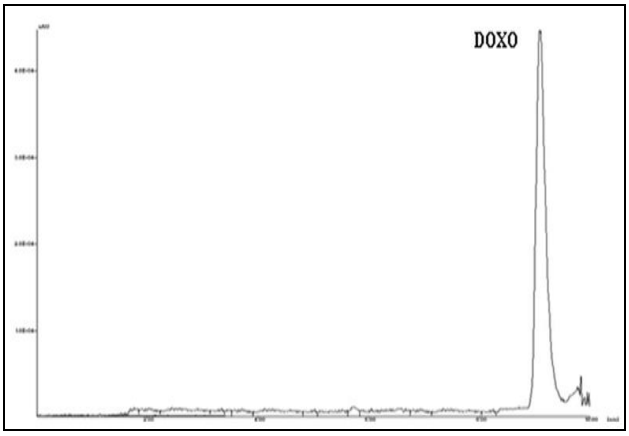

FIG. 12: CHROMATOGRAM OF DOXO AFTER EXPOSING TO DRY HEAT

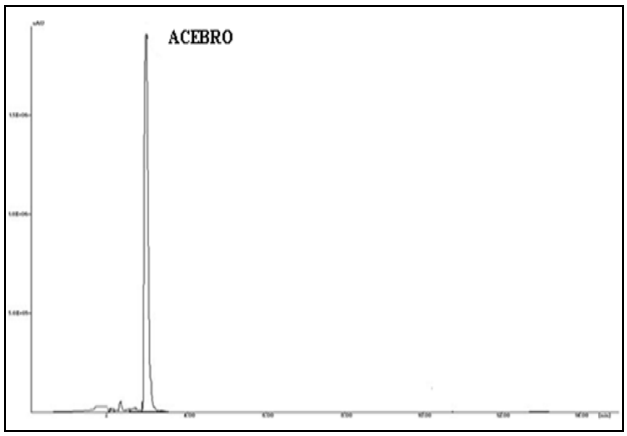

FIG. 13: CHROMATOGRAM OF ACEBRO AFTER PHOTODEGRADATION

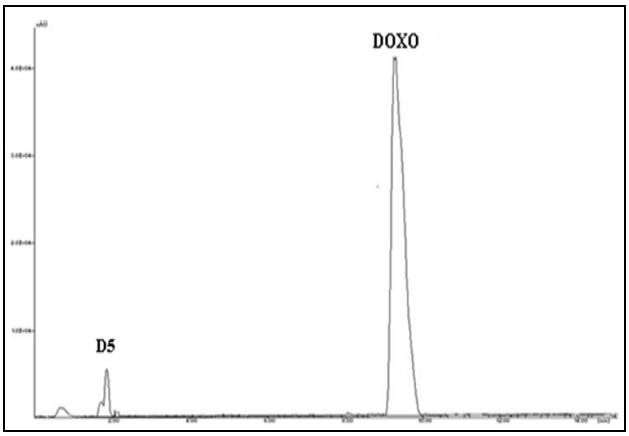

FIG. 14: CHROMATOGRAM OF DOXO) AFTER PHOTODEGRADATION (D5= 1.93 MIN

RESULT AND DISCUSSION: For selecting the mobile phase, initial trials were carried out using methanol and acetonitrile in various proportions and a buffer of varying pH to obtain the desired system suitability parameters. After several trials, finally, Acetonitrile and 10 mM n-hexane sulfonic acid buffer in the ratio of (80: 20, v/v) was selected as a mobile phase which provides symmetrical peaks using hypersil C18 column. Under the mentioned chromatographic conditions, highly symmetrical and sharp peaks of Acebrophylline and Doxofylline were obtained at retention times of 2.77 and 9.56 min, respectively **Fig. 3.** System suitability data are given in **Table 5.**

Stress Degradation Study of ACEBRO and DOXO:

TABLE 6: SUMMARY OF STRESS DEGRADATION STUDY OF ACEBRO AND DOXO

Agent	Exposure time (hr)	Number of Degradation products (Retention time in a minute)		% of drug remaining after degradation	
		ACEBRO	DOXO	ACEBRO	DOXO
HCl (0.1N)	0.5	1 (1.8)	1 (5.82)	85.16	91.81
NaOH (0.1 N)	0.5	1 (2.51)	1 (1.91)	89.83	88.43
Water	0.5	No degradation	1 (7.30)	90.50	87.26
H_2O_2 (30%)	0.5	No degradation	1 (1.92)	88.66	91.62
Dry Heat	1	No degradation	No degradation	100.16	90.43
Photo degradation	4	No degradation	1 (1.93)	94.55	88.98

The developed chromatographic method was validated using ICH guidelines. Validation parameters tested include linearity, accuracy, precision, robustness, specificity, the limit of detection, and quantitation. Linear calibration plots for the proposed method were obtained in

concentrations ranges of 1-10 µg/ml for acebrophylline and 4-24 µg/ml for doxofylline. The linear regression equation for acebrophylline was y = 14317 × + 96805 with a correlation coefficient greater than 0.997. The linear regression equation for doxofylline was found to be y = 37618 ×13096, with a correlation coefficient greater than 0.998. The limit of detection (LOD) and quantitation (LOQ) was determined by making serial dilutions. LOD was found to be 0.56 µg/ml and 0.44 µg/ml for Acebrophylline and doxofylline, respectively (signal to noise ratio of 3:1). LOQ was found to be 1.70 µg/ml and 1.33 µg/ml for Acebrophylline and doxofylline, respectively (signal to noise ratio of 10:1). Accuracy of the developed method was performed by standard addition method .three levels of solution (80%, 100%, and 120%) of the nominal analytical concentrations were prepared and analyzed by the developed method. Percent recoveries along with standard deviation and relative standard deviations for each analyte are given in **Table 2.**

Recovery studies showed the method to be highly accurate and suitable for the intended use. Intraday precision was determined by injecting three standard solutions of three different concentrations on the same day, and inter-day precision was determined by injecting the same solutions for three consecutive days. The relative standard deviation (RSD %) of the peak area was calculated to represent precision. Results of intra-day and inter-day precision are presented in **Table 1.** The robustness of the method was performed by slightly varying chromatographic conditions. The results showed that slight variations in chromatographic conditions had a negligible effect on the chromatographic parameters. Results are presented in **Table 4**. The specificity of the developed method was evaluated by applying different stress conditions (acid, base, oxidative, neutral, thermal and photolytic). The chromatograms under different stress conditions are shown in **Fig. 4, 14.** The results of stress studies are given in **Table 6.** All the stress conditions applied were enough to degrade both the drugs. Comparison of the two drugs showed that Acebrophylline is more stable as compared to doxofylline. Under acidic conditions, acebrophylline was degraded up to 14.84%, and doxofylline was degraded up to 8.19%. Under basic stress acebrophylline was degraded up to 10.17%, and doxofylline was degraded up to 11.57%. Under neutral stress, acebrophylline was degraded up to 9.5% and doxofylline was degraded up to 12.74%. Under dry heat stress conditions acebrophylline was stable, and doxofylline was degraded up to 9.57%. Under photolytic stress, acebrophylline and doxofylline were degraded up to 5.4% and 11.02%, respectively. From these stress studies, it is concluded that acebophylline was stable only to dry heat conditions while unstable to rest of the conditions. Doxofylline is not stable under all the stress conditions.

CONCLUSION: This study presents a simple and validated stability-indicating HPLC method for estimating Acebrophylline and Doxofylline in the presence of degradation products. The developed method was accurate, precise, sensitive, specific, rapid, and robust. The method is good enough to separate the peaks of active pharmaceuticals ingredients from the degradation products produced during forced degradation studies. Statistically, analysis proves that there were no statistically significant differences between developed methods. The developed method can be used as a quality-control tool for routine quantitative analysis of Acebrophylline and Doxofylline.

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CONFLICTS OF INTERESTS: Declared none

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