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DEVELOPMENT AND VALIDATION FOR SIMULTENEOUS ESTIMATION OF AMBROXOL HYDROCHLORIDE AND DOXOFYLLINE IN PHARMACEUTICAL DOSAGES FORMS AND BULK DRUGS BY RP-HPLC METHOD

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ABSTRACT

The present study described a new, simple, accurate, and precise development of RP-HPLC method for the simultaneous Estimation of Ambroxol and Doxofylline in bulk and tablet dosage form. The chromatographic method was standardized using a ODS Inertsil C18, 250 mm × 4.6 mm, 5µ (particle size), with gradient conditions and mobile phase containing potassium dihydrogen orthophosphate buffer-pH 3.5 (0.01M KH2PO4): acetonitrile (60:40) at flow rate of 0.8ml/min using UV detection at 257nm. The retention times of Ambroxol and Doxofylline were 3.446 min and 4.546 min, respectively. The method was linear over the concentration range for Ambroxol 3.75-22.50µg/ml and for Doxofylline 50-300µg/ml. The recoveries of Ambroxol and Doxofylline were found to be in the range of 99.18 to 99.26% and 99.19 to99.39% respectively. The validation of method was carried out utilizing ICH guidelines. The described HPLC method was successfully employed for the analysis of bulk and pharmaceutical dosage form.

KEY WORDS

Ambroxol, Doxofylline, Simultaneous estimation, HPLC, Stability.

1. INTRODUCTION

There are many methods on development and validation for simultaneous estimation of ambroxol hydrochloride and doxophylline in pharmaceutical dosage forms and bulk drugs by rp-hplc method. Hence alternate methods for existing products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. Here the study includes validation parameters and stability studies effecting by temperature. This method can be used for the analysis of formulation development and stability testing to some extent in the quality control laboratory use.

In the proposed work, attempt shall be made to:

1. Develop new analytical method for the simultaneous estimation of Ambroxol and Doxofylline by HPLC.
2. Validate the method as per ICH guide lines.
3. In the literature survey it was found that Doxofylline and Ambroxol are estimated independently and in combination with other drugs by several Bioanalytical, HPLC and Spectrometric methods.
4. In the view of the need in the industry for routine analysis of Doxofylline and Ambroxol in formulation, attempts are being made to develop simple and accurate analytical methods for simultaneous estimation of Ambroxol and Doxofylline extend it for their determination in formulation.

DRUG PROFILE:[1-8]

Doxophylline is a white crystalline powder and odorless, slightly soluble in water, sparingly soluble in ethanol and methanol and is used as antitussive, anti-asthamatic drug. Doxofylline is indicated for the treatment of bronchial asthma and chronic obstructive pulmonary disease (COPD) in adults. Doxofylline's mechanism of action is related to the inhibition of phosphodiesterase activities similar to theophylline.

Ambroxol hydrochloride is a white crystalline powder, freely Soluble in acetone and methylene chloride, Very slightly soluble in water, soluble in chloroform

and methanol. Used as mucolytic agent and it stimulates production of pulmonary surfactant, a substance found to play a major role in the lung host defense mechanism, thereby further protecting against lung inflammation and infection; also exhibits anti-inflammatory and antioxidant activity. When administered orally, onset of action occurs after about 30 minutes.

2. MATERIALS AND METHODS

Chemicals and reagents:

The working standards of Ambroxol and Doxofylline were generous gift obtained from Richer Pharmaceuticals. Pvt. Ltd, prashanthi nagar, kukatpally,Hyderabad, and Telangana, India. The combination formulation of Ambroxol and Doxofylline (Synasma-AX) marketed by Ranbaxy Laboratories Ltd., India.) Tablets were purchased from the local market. Acetonitrile, methanol, and water were used of HPLC grade make- Merck, Rankem. Potassium dihydrogen phosphate and phosphoric acid used were of analytical grade.

HPLC instrumentation:

Chromatographic separation was performed with waters (e2695, WATERS,)
having following components: LC-20AT Pump, waters 2489 UV/VisibleDetector, Inertsil ODS-3V C18, 250 mm × 4.6 mm, 5µ (particle size),Thermo scientific, Rheodyne auto Injector (20µlCapacity), Hamilton Syringe (25µl) and Chromatograms and data were recorded by means of EM power-2 Software.

Instruments used:

Digital balance- Sartorius CP224S.
PH meter- ELICO L1 120.
UV-Visible Spectrophotometer- Shimadzu (UV- 1601), Sonicator- Ultra wave.

3. OPTIMISED CHROMATOGRAPHIC CONDITIONS

A simple, stable, accurate, precise, less time consumable, cheap and more economical process using RP-HPLC was developed for simultaneous estimation of Doxofylline and Ambroxol Hcl in bulk and Pharmaceutical dosage forms on C18 Inertsil 5µ, 250mm×4.6mm column using phosphate buffer (0.01M PH 3.5): ACN (60:40) as mobile phase the flow rate was 0.8ml/min and the effluent was monitored at 257nm.

CHROMATOGRAPHIC CONDITIONS:

Mobile phase : phosphate buffer (0.01M PH 3.5): ACN (60:40)
Column : C18 Inertsil ODS-3V, 5µm, (250mm×4.6mm)
Column temperature: 30° C
Flow rate : 0.8ml/min
Detection : 257nm
Injection volume : 20µl
Run time : 10 min
Diluents : Mobile Phase

STANDARD SOLUTION PREPARATION:

Transfer an accurately weighed quantity of about 3mg of Ambroxol HCl working Standard and 40mg of Doxofylline working standard in to 100ml volumetric flask add 75ml of Mobile phase and sonicate to dissolve the content, and make up to the volume with mobile phase and further dilute 10ml in to 100 ml with diluents, mix.

SAMPLE SOLUTION PREPARATION:

Accurately weigh and transfer powdered tablet Equivalent to 3 mg of Ambroxol Hcl [82mg] and 40mg of Doxofylline [82mg] into a 100ml clean dry volumetric flask add about 75ml of mobile phase and sonicate to dissolve it completely and make volume up to the mark with the mobile phase. Further dilute 10ml in to 50 ml with mobile phase, mix.

PROCEDURE:

Inject 20µL of the standard, sample into the chromatographic system and measure the areas for the Ambroxol Hcl and Doxofylline peaks and calculate the %Assay by using the formula.

4. VALIDATION OF PROPOSED METHOD:[9-13]

Method validation:

The developed analytical method was subjected to validation with respect to various Parameters such as linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, recovery studies, specificity and reproducibility and robustness / Ruggedness as per the ICH guidelines.

(a) SYSTEM SUITABILITY:

System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. According to USP system suitability are an integral part of chromatographic methods. These tests verify that the resolution and reproducibility of the system are adequate for the analysis to be performed.

- The RSD for the peak area of Doxofylline and Ambroxol hydrochloride for 5 replicate Injections should not be more than 2%.
- Tailing Factor for Doxofylline and Ambroxol hydrochloride should be not more than 2.

(b) LINEARITY:

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to concentration of analyte in samples. The range of an analytical is the intervals between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated. These characteristics are determined by application of the procedure to a series of Samples having analyte concentration spanning the claimed range of procedure. Hence for determination of Linearity six different concentrations are prepared and chromatograms were recorded for same.

Preparation of stock solution:

Transfer an accurately weighed quantity of about 3mg of Ambroxol HCl working standard and 40mg of Doxofylline working standard in to 100ml volumetric flask add 75ml of Mobile phase and sonicate to dissolve the content, and make up to the volume with mobile phase.

Procedure: Different levels of preparations from I (25%)-VI (150%) were prepared and analysed. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis. Peak area) and calculate the correlation coefficient.

(c) ACCURACY (RECOVERY STUDIES):

The accuracy is the closeness of the measured value to the true value for the sample. The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels the specified range (i.e, three concentrations and three replicates of each concentration).

Accuracy was tested (% Recovery and % RSD of individual measurements) by analyzing samples at least in triplicate, at each level (50,100 and 150 % of label claim) is recommended. For each determination fresh samples were prepared and assay value is calculated. Recovery was calculated from regression equation obtained in linearity study. Accuracy was determined from the mean relative error for a set of replicate analysis (i.e.the difference between measured and nominal concentration) for spiked samples.

Standard Solution Preparation:

Transfer an accurately weighed quantity of about 3mg of Ambroxol HCl working standard and 40mg of Doxofylline working standard in to 100ml volumetric flask add 75ml of Mobile phase and sonicate to dissolve the content, and make up to the volume with mobile phase and further dilute 10ml in to 100 ml with mobile phase , mix.

Procedure: sample solutions of different concentrations of 50,100,150% were prepared with respect to target assay concentrations and injected the standard solution, Accuracy -50 %, Accuracy -100 % and Accuracy -150 % solutions.

(d) PRECISION:

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed the variance, standard deviation of coefficient of variation of a series of measurement.

Preparation of stock solution:

40mg of Doxofylline working standard in to 100ml volumetric flask add 75ml of Mobile phase and sonicate to dissolve the content, and make up to the volume with mobile phase and further dilute 10ml in to 100 ml with mobile phase , mix.

Procedure:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

INTERMEDIATE PRECISION

To evaluate the intermediate precision of the method,Precision was performed on different day by using different make column of same dimensions.

Preparation of stock solution:

Transfer an accurately weighed quantity of about 3mg of Ambroxol Hcl working standard and 40mg of Doxofylline working standard in to 100ml volumetric flask add 75ml of Mobile phase and sonicate to dissolve the content, and make up to the volume with

mobile phase and further dilute 10ml in to 100 ml with mobile phase , mix.

Procedure:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

(e) LIMIT OF DETECTION (LOD):
The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

1. Based on Signal-to-Noise - This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between3:1 is generally considered acceptable for estimating the detection limit.

2. Based on the Standard Deviation of the Response and the Slope:
The detection limit (DL) may be ex pressed as:
DL = 3.3 s/S
Where,

s = the standard deviation of y-intercepts of regression lines
S = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte.

(f) LIMIT OF QUANTITATION (LOQ):
The quantization limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

The quantization limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.
Several approaches for determining the quantization limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified.

1. A typical signal-to-noise ratio is 10:1.
2. Based on the Standard Deviation of the Response and the Slope

The quantization limit (QL) may be expressed as:
QL = 10 s/S
Where, s = the standard deviation of the response; S = the slope of the calibration curve

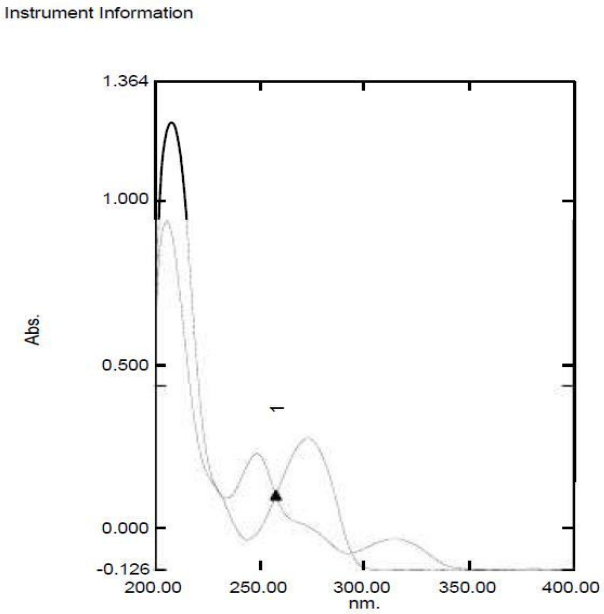
(g) RUGGEDNESS:
Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst. The Ruggedness was determined by using the data obtained by the analysis performed by two different analysts. Each analyst prepared 5 samples of the same batch and the results obtained.

(h) ROBUSTNESS:
Robustness of an analytical method is measure of its capacity to remain unaffected small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.
The flow rate was varied at 0.6 ml/min to 1ml/min.
The actual flow rate is 1.0 ml/min. Change the flow rate ± 0.2 ml/min and observed USP tailing and USP plate count.
The mobile phase composition was changed: Actual mobile phase ratio was buffer: ACN is 60:40
It was changed to 65:35 and 55:45 and observed USP tailing and USP plate count.

RESULTS AND DISCUSSION

5. DETERMINATION OF λMAX FOR DOXOFYLLINE AND AMBROXOL HCL BY UV.

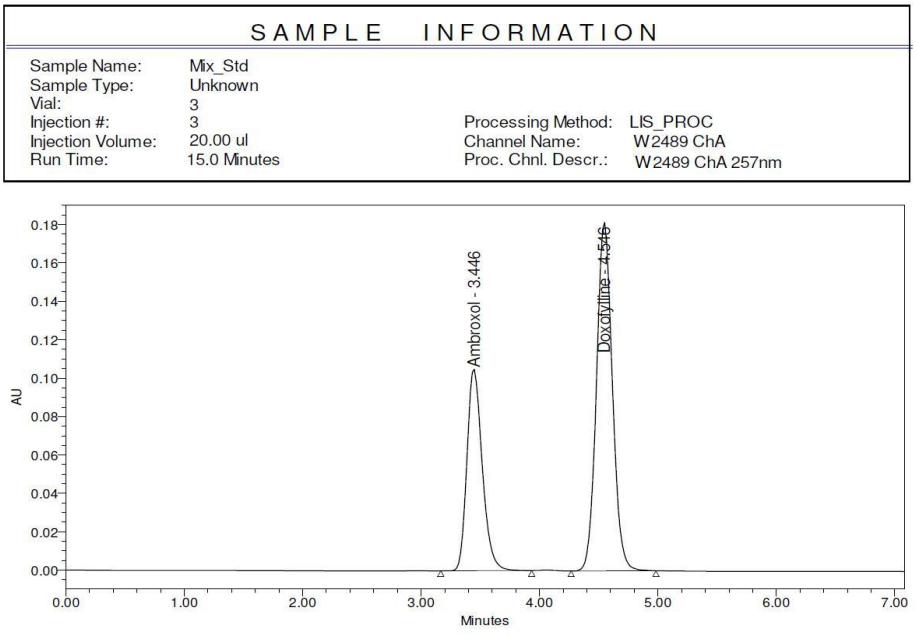
Fig.1. UV Graph of Ambroxol and Doxofylline



No.	Wavelength	Absorbance	Description
1	257.80	0.199	Ambroxil&Do
2			

5.1 OPTIMIZED TRIAL:

Fig.2. Chromatogram of Optimized Trial



	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1	Ambroxol	3.446	948408	104993	35.95		1.23	3410
2	Doxofylline	4.546	1689444	181392	64.05	4.63	1.07	5544

Observation: Peaks are well separated; Plate count, resolution and symmetric factors are within the acceptable limits. So, the method is finalized.

5.2 DATA FOR SYSTEM SUITABILITY:

Table.1: Data for System Suitability for Ambroxol Hydrochloride:

S No		Ambroxol
	RT	Area
1	3.486	1239517
2	3.486	1241754
3	3.486	1246030
4	3.486	1244401
5	3.485	1247129
6	3.486	1251757
Average	3.486	1245098
Standard Deviation	0.0004	4293.1
%RSD	0.0117	0.345

Table.2: DATA FOR SYSTEM SUITABILITY FOR DOXOFYLLINE:

S No	Doxofylline	
	RT	Area
1	4.313	4209541
2	4.312	4212874
3	4.312	4232293
4	4.312	4228294
5	4.311	4250605
6	4.311	4248839
Average	4.312	4230408
Standard Deviation	0.001	17311.94
%RSD	0.02	0.41

5.3 LINEARITY RESULTS OFAMBROXOL:

Fig.3: Linearity graph for Ambroxol HCl

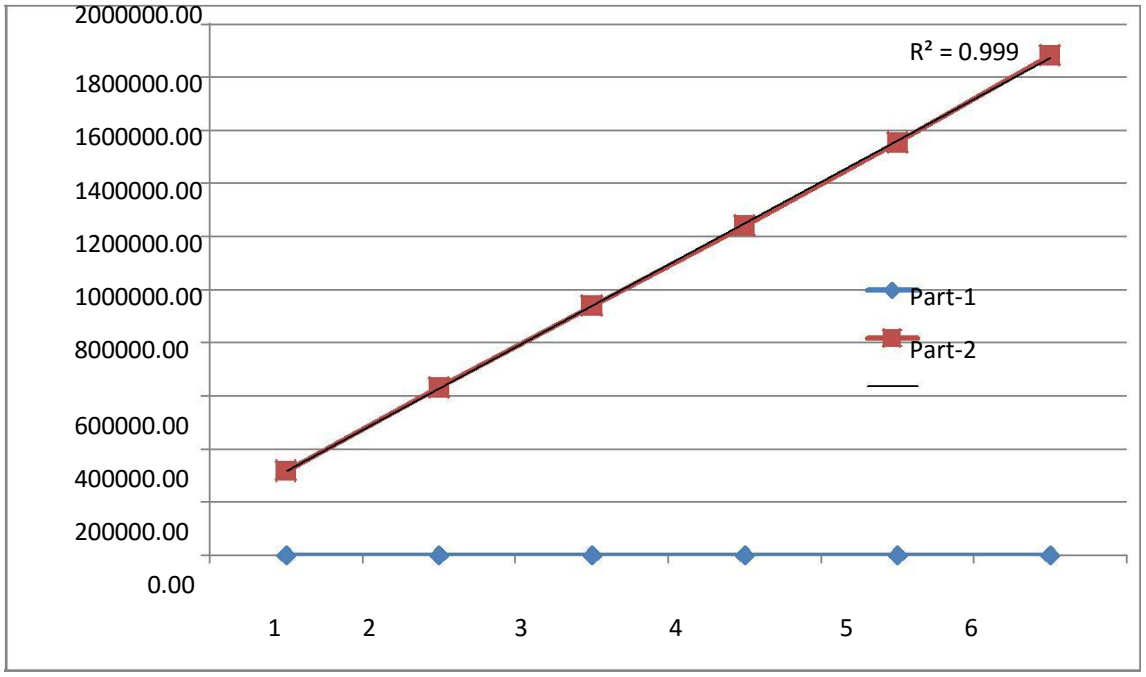


Table.3: Linearity results for Ambroxol:

Conc. (mcg)	Area
3.75	318579
7.50	632307
11.25	940714
15.00	1242080
18.75	1555290
22.50	1882846
Correlation coefficient	0.999

5.4 LINEARITY RESULTS OF DOXOFYLLINE:

Fig.4: Linearity graph for Doxofylline

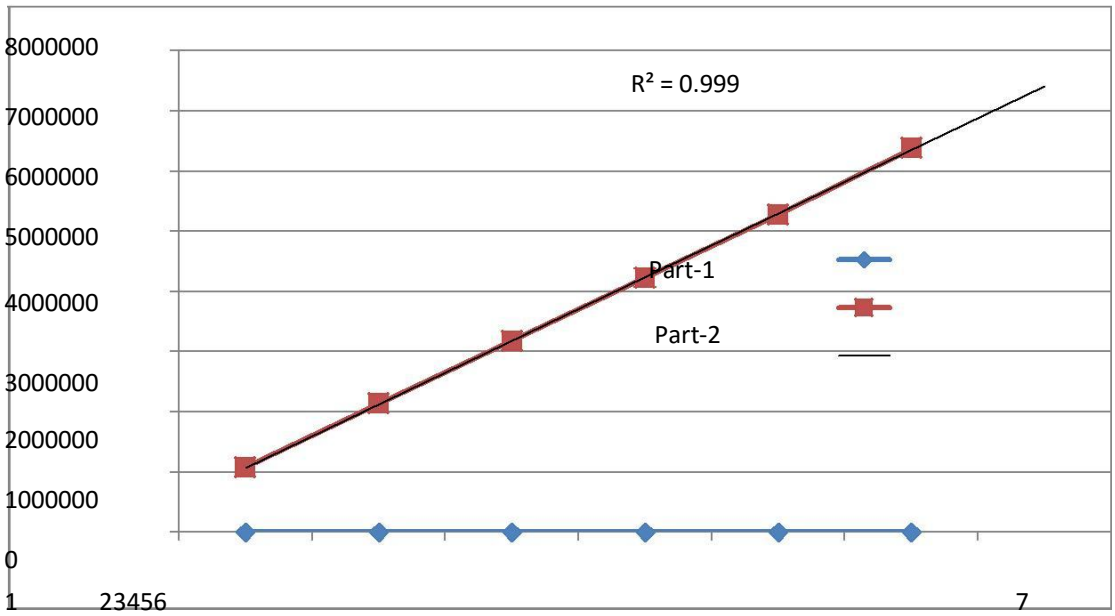


Table.4: Linearity results for Doxofylline

Conc.(mcg)	Area
50	1083182
100	2140868
150	3178742
200	4234699
250	5283960
300	6383864
Correlation coefficient	0.999

Acceptance criteria:

Correlation coefficient for 25% to 150% concentration should be NLT 0.999.

Inference:

The plot obtained for Concentration vs. Area was found to be linear and the correlation coefficient was found to be 0.999 for both Ambroxol HCl and

Doxofylline. Hence the method ensures that the results are directly proportional to concentration within the given range.

ACCURACY RESULTS:

Table.5: Accuracy Results of Ambroxol and Doxofylline

	Accuracy-- 50%		Accuracy-- 100%		Accuracy-- 150%	
	Ambro	Doxo	Ambro	Doxo	Ambro	Doxo
S No	**Area**	**Area**	**Area**	**Area**	**Area**	**Area**
Injection-1	989546	3393262	1238232	4222121	1495695	5072521
Injection-2	993025	3353232	1247565	4220120	1488513	5035654
Injection-3	994545	3366565	1239665	4219232	1477756	5091236
Avg amt	**992372**	**3371020**	**1241821**	**4220491**	**1487321**	**5066470**
Recovered	**79.41**	**79.35**	**99.20**	**99.32**	**119.02**	**119.27**
%Recovery	**99.26**	**99.19**	**99.20**	**99.32**	**99.18**	**99.39**

Acceptance criteria:
% Recovery at each level should be in between 98% to 102%.
Inference:
The results obtained for recovery at 50%, 100%, 150% were found to be within in the limits. Hence the proposed method was found to be accurate.

5.6 PRECISION:

Precision of the method was determined as described under experimental work and the corresponding chromatograms and results are shown below:

5.6.1 METHOD PRECISION RESULTS:

Table.6: Method Precision results for Ambroxol and Doxofylline

S No	Name	Ambroxol		Doxofylline	
		RT	Area	RT	Area
1	M-Precision-1	3.481	1220596	4.323	4201252
2	M-Precision-2	3.477	1226595	4.319	4199998
3	M-Precision-3	3.489	1230155	4.315	4222215
4	M-Precision-4	3.485	1229899	4.316	4201213
5	M-Precision-5	3.486	1221999	4.315	4215222
6	M-Precision-6	3.488	1245656	4.316	4212121
	Average	3.484	1229150	4.317	4208670
	Standard Deviation	0.0045	8998.4	0.003	9210.24
	%RSD	0.1305	0.732	0.07	0.22

Acceptance criteria: %RSD for sample should be NMT 1.
Inference: The %RSD for the sample solution was found to be below 1, Hence method is said to be precise.

5.6.2 SYSTEM PRECISION RESULTS:

Table.7: System Precision Results for Ambroxol and Doxofylline.

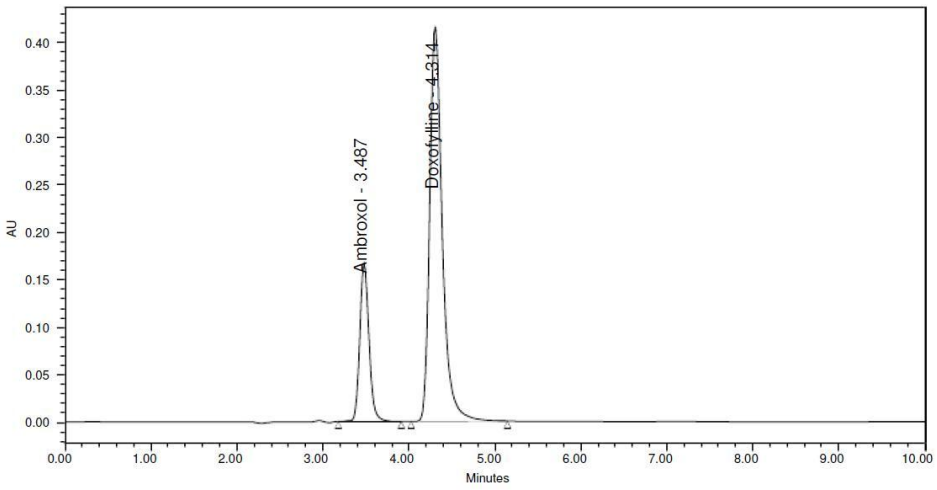
S No	Name	Ambroxol		Doxofylline	
		RT	Area	RT	Area
		3.486	1239517	4.313	4209541
1	S-Precision-1				
		3.486	1241754	4.312	4212874
2	S-Precision-2				
		3.486	1246030	4.312	4232293
3	S-Precision-3				
		3.486	1244401	4.312	4228294
4	S-Precision-4				
		3.485	1247129	4.311	4250605
5	S-Precision-5				
		3.486	1251757	4.311	4248839
6	S-Precision-6				
	Average	3.486	1245098	4.312	423408
	Standard Deviation	0.0004	4293.1	0.001	17311.94
	%RSD	0.0117	0.345	0.02	0.41

Acceptance criteria: %RSD for sample should be NMT 1.

Inference: The %RSD for the sample solution was found to be below 1, Hence method is said to be precise.

5.7 SPECIFICITY:

Fig.5: Chromatogram for Specificity of Standard-1



	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1	Ambroxol	3.487	1232691	167875	22.65		1.05	5856
2	Doxofylline	4.314	4220012	416589	77.35	3.89	1.03	4986

Fig.6: Chromatogram for Specificity of Placebo

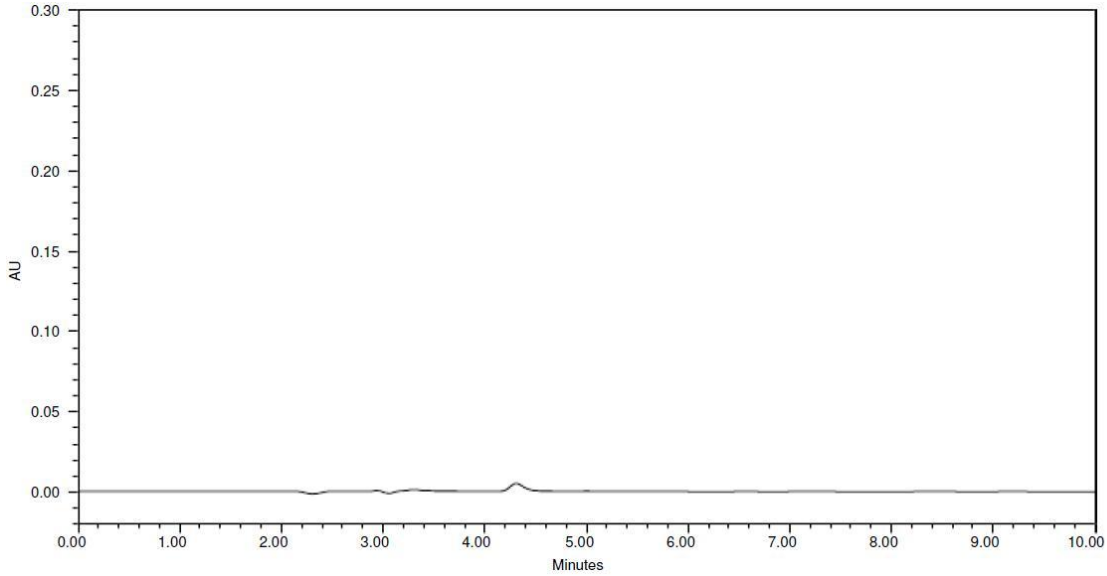
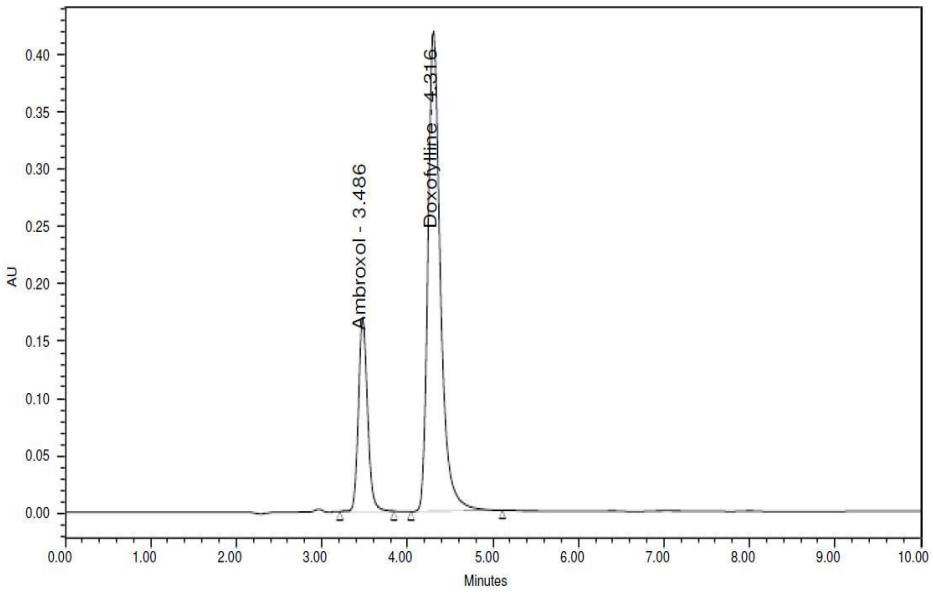


Fig.7: Chromatogram for Drug sample after Placebo



	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1	Ambroxol	3.486	1219652	168784	22.67		1.05	5351
2	Doxofylline	4.316	4224152	419452	77.33	3.89	1.07	4745

RESULT: There is no peak merged with drug peak and the peaks are separated so the method is said to be specific.

5.8 ROBUSTNESS:

Table.8: Robustness results for Ambroxol and Doxofylline

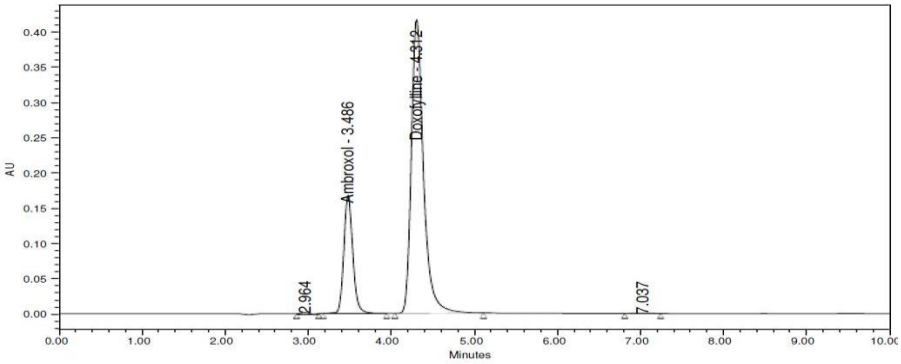
S.No.	Ambroxol		Doxofylline	
	RT	Area	RT	Area
		Standard		
1	3.487	1232691	4.314	4220012
		Robust-1 Flow -1		
2	3.107	1122733	3.841	3791183
		Robust-2 Flow-2		
3	3.971	1434649	4.915	4879613
	Robust-3 Column Oven Temperature-1			
4	3.506	1253860	4.338	4252684
	Robust-4 Column Oven Temperaure-2			
5	3.467	1250755	4.304	4273151

Result:

On evaluation of the above results, it can be concluded that the variation in flow rate and temperature not much affected the test results. Hence, it indicates that the method is robust even by change in the flow rate ±20%.

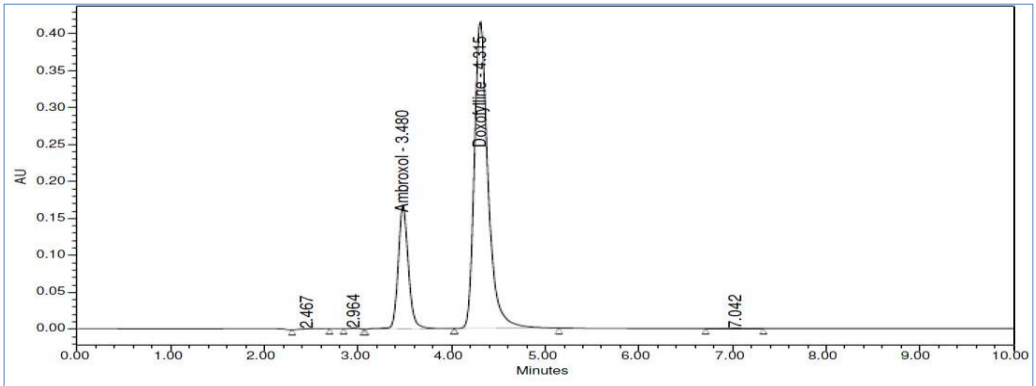
5.9 STABILITY STUDIES:

Fig.8. Chromatogram for Stability Indicting at 5°C



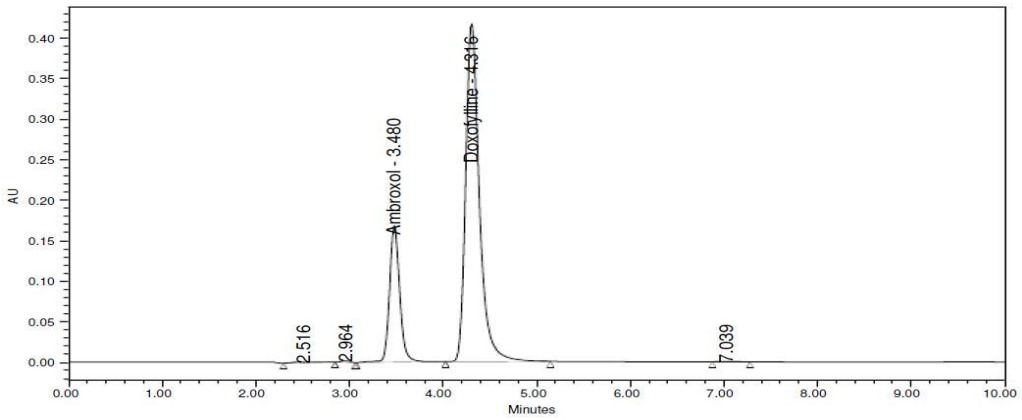
	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1		2.964	10158	1512	0.19		0.89	7093
2	Ambroxol	3.486	1204401	167541	22.67	3.15	1.16	5211
3	Doxofylline	4.312	4218294	417466	77.04	3.75	1.16	4589
4		7.037	5416	441	0.10	9.35	0.99	7091

Fig.9. Chromatogram for Stability Indicating At 25º c/60% RH



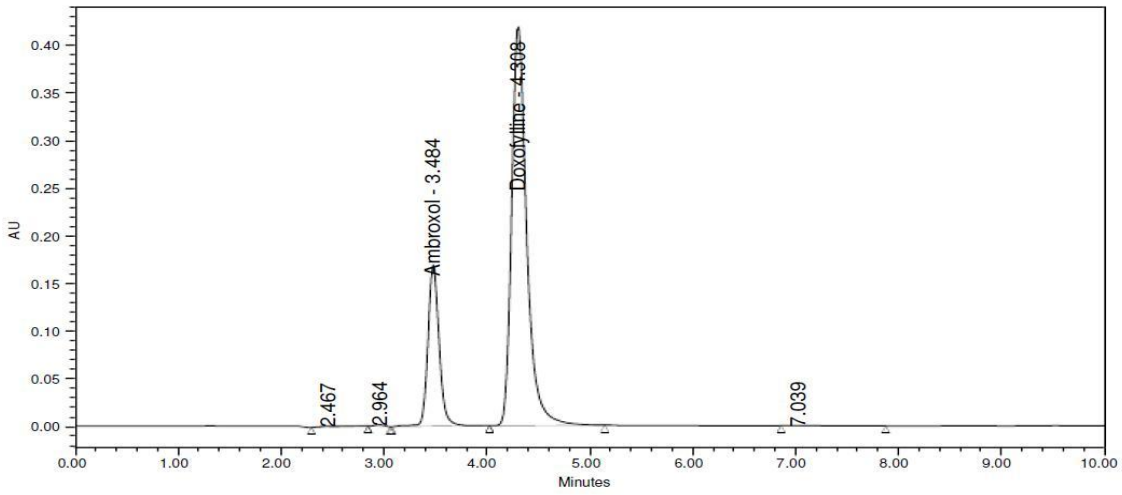
	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1		2.467	10088	757	0.18		0.59	2112
2		2.964	10158	1699	0.18	2.96	1.04	5367
3	Ambroxol	3.480	1186962	167709	23.02	2.99	1.14	5199
4	Doxofylline	4.315	4125875	415931	76.50	3.75	1.36	4604
5		7.042	6127	459	0.11	9.15	1.02	6504

Fig.10. Chromatogram for Stability Indicating At 40^{0}C/75%RH



	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1		2.516	16545	865	0.30		1.27	2338
2		2.964	10843	1827	0.20	1.12	1.04	5450
3	Ambroxol	3.480	1214391	168278	23.01	3.00	1.14	5176
4	Doxofylline	4.316	4222293	417437	76.42	3.74	1.36	4589
5		7.039	4195	375	0.08	9.74	1.22	8442

Fig.11. Chromatogram for Stability Indicating At Room TEMP (25°C)



	Peak Name	RT	Area	Height	% Area	Resolution	Symmetry Factor	USP Plate Count
1		2.467	16911	924	0.30		0.54	2211
2		2.964	11345	1921	0.20	3.04	1.04	5495
3	Ambroxol	3.484	1227345	168970	22.97	3.00	1.14	5185
4	Doxofylline	4.308	4220325	419359	76.43	3.74	1.16	4585
5		7.039	5539	424	0.10	8.95	1.19	7234

CONCLUSION

An accurate assay method was developed for the determination of ambroxol hydrochloride and doxofylline by using RP-HPLC method.

The surveillance and outcome obtained from each from each validation experiment including specificity, linearity range, LOD and LOQ, precision, accuracy, robustness, ruggedness stability indicating on temperature studies and system suitability lies well inside the acceptance criteria. Since, all the results are within the limit, the developed analytical method is considered as validated and suitable for anticipated use and all parameters are subjected as per the ICH guidelines.

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