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Development and validation of RP-HPLC method for stability testing of Roxithromycin and Ambroxol hydrochloride

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ABSTRACT

RP-HPLC method was developed for simultaneous estimation of Roxithromycin and Amroxol hydrochloride in pharmaceutical dosage forms. The method was validated for the simultaneous estimation of Roxithromycin and Ambroxol hydrochloride in combined pharmaceutical table dosage form. The RP-HPLC method was developed by using BDS Hypersil C18 column; (150×4.6×5µ) column at 254nm, flow rate of 1.0ml/min., Injection volume of 10µl, column oven temperature of 250 C using equal volume of acetonitrile and 0.05M Potassium dihydrogen orthophosphate pH-3 adjust with orthophosphoric acid was used as mobile phase(50:50v/v). The retention times were found to 2.830 and 4.047 mints. The % purity was found to be 99.47 and 99.97% w/w respectively.The analytical method was validated according to ICH guidelines (ICH, Q2 (R1).The correlation coefficient (r2) was found to be 0.998 respectively, % recovery was 99%, %RSD for system precision was found to be0.6576, 0.3466 and for repeatability was 0.53 ,0.403 respectively.

1. INTRODUCTION

Ambroxol is an active N-desmethyl metabolite of the mucolytic, bromhexine. Ambroxol is shown to exert several activities, as follows: Its mucolytic activity by which it facilitates breakdown of acid mucopolysaccharide fibres in the mucous making it thinner and less viscous and, therefore, easy for expectoration; it stimulates the ciliary activity thereby improving mucokinesis (transport of mucous); 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.

Roxithromycin prevents bacteria from growing, by interfering with their protein synthesis. Roxithromycin binds to the subunit 50s of the bacterial ribosome, and thus inhibits the translocation of peptides. Roxithromycin has similar antimicrobial spectrum as erythromycin, but is more effective against certain gram-negative bacteria, particularly Legionella pneumophila.

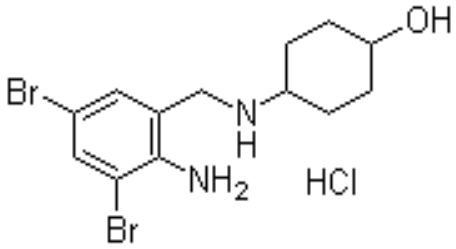

Figure.1.Chemical structure of Ambroxol Hydrochloride

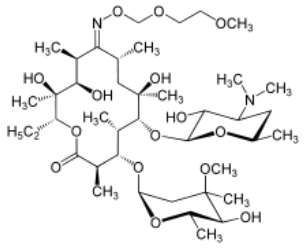

Figure.2.Chemical structure of Roxithromycin

2. MATERIALS AND METHODS

Ambroxol hydrochloride working standard, Roxithromycin working standard, RoxiRT tablets procured from Spectrum Pharma research solutions. Potassium dihydrogen phosphate, Acetonitrile, Orthophosphoric acid (88%), Methanol was purchased from Merck.

Selection of column: Column of HPLC made of 316 grade stainless steel packed with stationary phase. In reverse phase chromatography, Hydrophobic interaction between drug molecule and the alkyl chains on the column packing material. Column is selected based on solubility, polarity & chemical differences among analytes. BDS Hypersil C_{18} column (150 × 4.6 mm, 5µm) was selected, because it has better separation, and good tailing factor.

Selection of mobile phase: To develop a precise and robust HPLC method for determination of Roxithromycin and Ambroxol hydrochloride, their standard solutions were injected in the HPLC system. After literature survey and solubility data different composition of mobile phase of different flow rates were employed in order to determine the best condition for effective separation of both drugs.

Selection of chromatographic method: The reversed phase HPLC was selected for the initial separation because of its simplicity and suitability. From the literature survey and with the knowledge of properties of the selected drug, BDS hypersil C_{18} (150 X 4.6 mm) 5µ column was chosen as stationary phase and mobile phase potassium dihydrogen phosphate pH-3 adjusted with ortho phosphoric acid and acetonitrile with the ratio of 50:50 v/v was used. From all the data observed, obtained and available the initial separation conditions were set to work around.

Selection of mode of separation: The selection of method depends upon the nature of the sample, its molecular weight and solubility. The drug selected in the present study is in polar nature and hence RP-HPLC method was preferred because of its simplicity and suitability. While developing RP-HPLC method with different mobile phases like Acetonitrile**:** water, Acetonitrile **:** Buffer (Acetate, Phosphate Buffer), and Methanol**:** water was used, but no favorable results obtained.

Method Optimization:

Initial chromatographic conditions:

Stationary phase: OEM C18 (250 mm x 4.6 mm i.d, 5µm)
Mobile phase : Water : Acetonitrile (40:60 v/v)
Flow rate : 0.5 ml / min
Detector wavelength : 254 nm
Column temperature : Ambient

Effect of flow rate on separation: The mobile phase consisting of Buffer: Acetonitrile was used and the chromatograms were recorded at flow rates of 0.5 ml, 1 ml, 1.5ml, and 2 ml. The sharpest peaks were obtained with 1ml flow rate.

Effect of ratio of mobile phase: To develop a precise and robust HPLC method for determination of Roxithromycin and Ambroxol hydrochloride, their standard solutions were injected in the HPLC system. After literature survey and solubility data different composition of mobile phase of different flow rates were employed in order to determine the best condition for effective separation of both drugs.

Procedure:

Preparation of Phosphate buffer: Weighed 3.4 g of Potassium di hydrogen phosphate into a 500ml beaker, dissolved and diluted to 500 ml with HPLC water. Adjusted the pH to 3 with orthophosphoric acid.

Preparation of mobile phase: Mix a mixture of above buffer 500 ml and 500 ml of Acetonitrile HPLC in the ratio of 50:50 (v/v) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation: Accurately 50 mg of Roxithromycin& 10 mg of Ambroxol hydrochloride working standard was transferred into a 50 ml clean dry volumetric flask. The compounds were first dissolved in 20 ml of mobile phase and it was sonicated to dissolve completely and the volume was make up to the mark with the same solvent. (Stock solution) From the stock solution 1ml was pipette out in to 10 ml volumetric flask and dilute up to the mark with mobile phase.

Trial method: 1

Chromatographic Conditions

Stationary phase: OES C_{18} column (250 mm x 4.6 mm i.d, 5µm)
Mobile phase : Water: Acetonitrile (85:15v/v)
Flow rate : 0.5 ml / min
Detector wavelength: 254 nm
Column temperature: Ambient
Injection volume: 10 µl

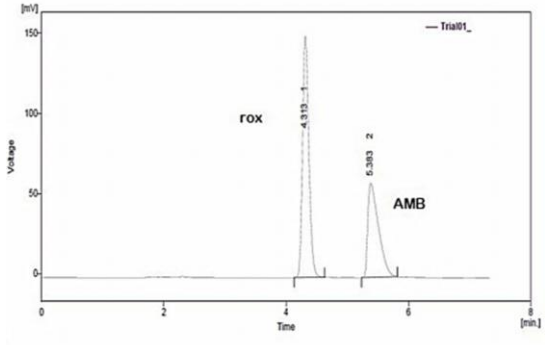

Figure.3.Shows the Chromatogram for trial 1

Observation: System suitability was failed. Fronting and tailing of the peaks was seen. The chromatogram is shown in Figure.3.

Trial method: 2

Chromatographic Conditions

Stationary phase: Inertisil ODS C_{18} column (250 x 4.6 mm i.d, 5µm)

Mobile phase : Water: methanol (20:80 v/v)
Flow rate : 1 ml / min
Detector wavelength : 254 nm
Column temperature : Ambient
Injection volume : 10 µl

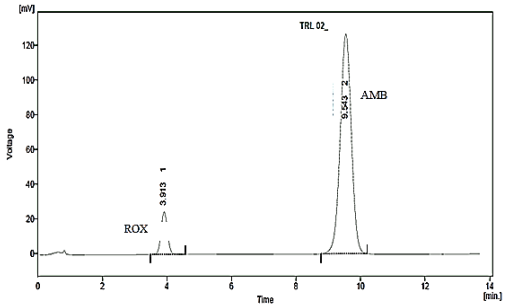

Figure.4.Shows the Chromatogram for trial 2

Observation: System suitability was failed. Fronting and tailing of the peaks was seen, retention time was more so another trail was performed. The chromatogram is shown in Figure.4.

Trial method: 3

Chromatographic Conditions

Mobile phase : Mixture of phosphate buffer (potassium dihydrogen phosphate & 0.003M dipotassium hydrogen phosphate: Acetonitrile (40:60 v/v) pH -6.8
Flow rate : 1 ml / min
Detector wavelength : 254 nm
Column temperature : Ambient
Injection volume : 10 µl
Run time : 25 min

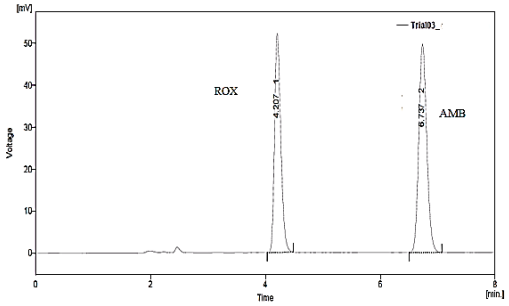

Figure.5.Shows the Chromatogram for trial 3

Observation: System suitability was failed. Fronting and tailing of the peaks was seen, retention time was more so another trail was performed. The chromatogram is shown in Figure.5.

Trial method: 4

Chromatographic Conditions:

Stationary phase: Inertsil ODS C_{18} column (250 x 4.6 mm i.d, 5µm)

Mobile phase : 0.01 M Sodium di hydrogen ortho phosphates Mixture: Acetonitrile
Flow rate : 1 ml / min
Detector wavelength : 254 nm
Column temperature : Ambient
Injection volume : 10 µl
Run time : 25 min

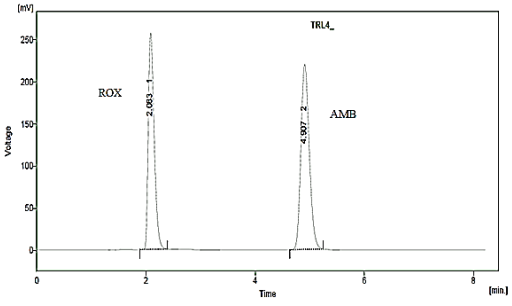

Figure.6.Shows the Chromatogram for trial 4

Observation: System suitability was failed. Fronting and tailing of the peaks was seen, slight fronting was observed so another trail was performed. The chromatogram is shown in Figure.6.

Optimized method:

Chromatographic Conditions:

Stationary phase : BDS Hypersil C_{18} column (250 x 4.6 mm i.d, 5µm)
Mobile phase : 0.05M potassium dihydrogen ortho phosphate pH-3 adjusted with orthophosphoric acid: Acetonitrile (50:50 v/v)
Flow rate : 1 ml / min
Detector wavelength : 254 nm
Column temperature : Ambient
Injection volume : 10 µl
Run time : 10 min

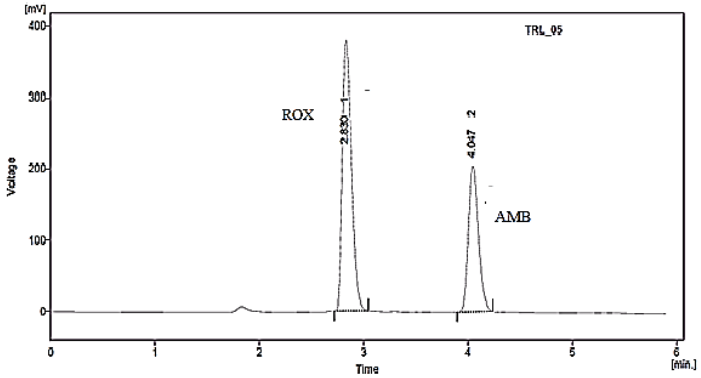

Figure.7.Shows the Chromatogram for optimized method

Resolution between two analytes is good. No peak asymmetry was observed. No other impurity interference was seen. All the results were found to be within the acceptance criteria. Hence the method was considered to be optimized.

3. RESULTS AND DISCUSSION

The objective of the proposed work was to develop a new method development and validation for the simultaneous estimation of Roxithromycin and Ambroxol hydrochloride in tablet dosage form by RP-HPLC.

Method development: Roxithromycin and Ambroxol hydrochloride was found to be soluble in methanol and isotonic solutions. The substance was dissolved in methanol to get a suitable concentration containing 100µg/ml and the resulting solution was scanned under UV region. The overlay spectrum of Roxithromycin and Ambroxol hydrochloride shows the isobestic point 254nm. Hence 254nm selected was maximum absorbance and the detection wavelength for the proposed method. In HPLC method, the condition was optimized to obtain an adequate elution of compounds. Initially, various mobile phase compositions was tried to separate the titled ingredients. Mobile phase, column selection, wavelength selection was based on peak parameters (height, tailing, theoretical plates, capacity or symmetry factor) and run time. The system with 0.05M potassium dihydrogen ortho phosphate salt and pH adjusted to 3 using orthophosphoric acid as buffer and using Buffer: Acetonitrile in the ratio of 50:50 (v/v) and a flow rate of 1.0 ml /min was found to be robust. The optimum wavelength for detection was 254 nm and a run time of 10 mins at which better detector for the drug along with no interference was obtained. The standard chromatograms were taken for the proposed method and various system suitable parameters were recorded.

The validated HPLC method was used for simultaneous determination of Roxithromycin and Ambroxol hydrochloride in their combined dosage form. In the assay experiment seven samples were weighed separately and analysed. The mean assay results expressed as a percentage of the label claim are listed in the table 1. The results indicate that the amount of each drug in the tablets is within the requirements of 90-110 % of the lable claim.

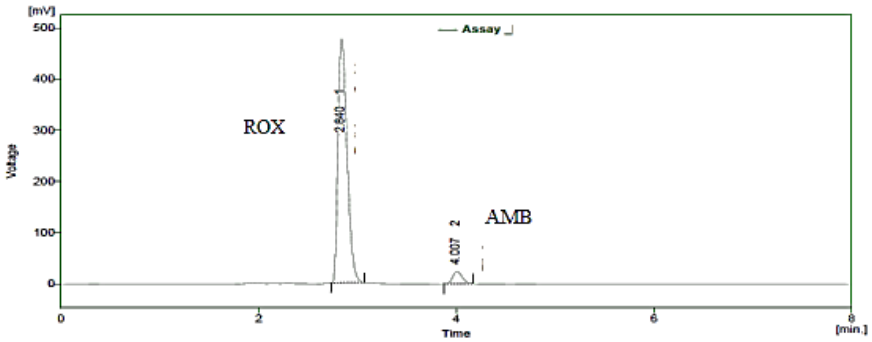

Figure.8.Shows the chromatogram for sample solution

Table.1.Calculations for assay of Roxithromycin and Ambroxol hydrochloride

	Roxithromycin	Ambroxol hydrochloride
Standerd area	3120.452	176.801
Sample area	3107.989	177.784
Standard wt	50.2mg	2.5mg
Sample wt	1015.3mg	1015.3mg
Avg wt	202.3mg	202.3mg
LC	10mg	0.5mg
% purity	99.4753	99.97928

Retention time was found to be 2.8 and 4.0 mins and the % purity of the Roxithromycin and Ambroxol hydrochloride was found to be 99.4753 % w/w & 99. 97928 % w/w respectively.

Method validation: The present study was carried method was validated based on ICH (Q2B) parameters. The following parameters were validated for the proposed method.

Specificity: Specificity of the method was found out through non-interference of the blank, internal standard and mobile phase. The method showed excellent specificity. The specificity study was performed for blank and the chromatograms shows that there is no interference in the retention time of the analyte.

Linearity: Calibration curve was constructed for Roxithromycin and Ambroxol hydrochloride standard by plotting the concentration of compound versus peak area response. Standard solutions containing 20, 40, 60, 80, 100 and 120 µg / ml of Roxithromycin and Ambroxol hydrochloride with respectively were prepared and 10µl was injected into the HPLC column the linearity was evaluated by linear regression analysis, which was calculated by the least square regression method on the ordinate. The result chromatograms are shown in the Fig. No. 28-35 and reports are tabulated in the Table 2 and 3.

Table.2.Calculations for linearity of Roxithromycin

Concentration µg/ml	Peak Area	Statical Analysis
20	560.978	
40	911.535	Slope: 20.59
60	1370.185	
80	1742.885	Intercept: 123.6
100	2224.455	
120	2581.977	Correlation coefficient: 0.998

Table.3.Shows the calculations for linearity of Ambroxol hydrochloride

Concentration µg/ml	Peak Area	Statical Analysis
1	34.805	
2	57.401	Slope: 26.04
3	83.692	
4	110.566	Intercept: 6.977
5	140.301	
6	161.984	**Correlation coefficient: 0.997**

The linearity study was performed for range of 20-120 µg and 1-6 µg. The correlation coefficient was found to be 0.998 and 0.997. (NMT 0.999)

Accuracy: The accuracy study was performed for 80 %, 100 % and 120 % for Roxithromycin and Ambroxol hydrochloride. Each level was injected in triplicate into chromatographic system. The area of each level was used for calculation of % recovery.

The accuracy study was performed for the range of 80 to 120. The % recovery of Roxithromycin and Ambroxol hydrochloride was found to be 99.5285% and 99.5306 % respectively (NLT-98 & NMT-102).

Precision:

Method precision: The reproducibility of the method was estimated by analyzing samples. Five injections of the standard mixture were analyzed for the determination of system precisions. Similarly five solutions of individual standard were prepared and assayed for the determination of method precision.

Table.4.Shows the peak results for accuracy of Roxithromycin and Ambroxol hydrochloride

	Roxithromycin	Ambroxol hydrochloride
Standard Conc.	50mg	2.5mg
Standard Area	3157.084 3143.527 3132.64 3148.886 3145.672	186.408 187.144 186.196 187.717 186.196
Avg	3145.562	186.7322
SD	8.87323	0.674451
%RSD	**0.282087**	**0.361186**

Roxithromycin:

Table.5.Shows the peaks results for method precision

S. No.	Retention Time	Area
1	2.843	2276.986
2	2.85	2271.488
3	2.847	2284.55
4	2.843	2268.708
5	2.843	2299.182
AVG	2.8452	2280.183
SD	0.003194	12.22224
% RSD	**0.11225**	**0.53602**

Ambroxol hydrochloride:

Table.6.Shows the peaks results for method precision

S. No.	Retention Time	Area
1	4.03	147.127
2	4.04	146.033
3	4.037	147.599
4	4.033	146.778
5	4.03	146.534
AVG	4.034	146.8142
STDEV	0.004416	0.592421
%RSD	**0.109467**	**0.403518**

System Precision: The system precision is checked to ensure that the analytical system is precise.

Table.7.Shows the peaks results for system precision

S.No.	Retention Time	Area
1	2.837	2301.053
2	2.837	2272.506
3	2.837	2268.978
4	2.853	2267.572
5	2.847	2264.088
AVG	**2.8422**	**2274.839**
SD	**0.00743**	**14.96129**
% RSD	**0.261406**	**0.657686**

Ambroxol hydrochloride:

Table.8.Shows the peaks results for system precision

S. No.	Retention Time	Area
1	4.003	145.964
2	4.003	146.645
3	4.003	146.372
4	4.043	145.307
5	4.033	145.959
AVG	**4.017**	**146.0494**
SD	**0.019494**	**0.506318**
% RSD	**0.485277**	**0.346676**

Intermediate Precision/Ruggedness: The intermediate precision study was performed for five injections prepared by two different analyst. Each standard injection was injected in to chromatographic system. The area of each standard injection was used for calculation of % RSD.

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Robustness: The method was evaluated for robustness by different parameters like change in buffer pH(±0.2 units), flow rate(±0.1ml/min) and detection wavelength(±5nm). Standard and check standard solutions were prepared as per the test method and injected into HPLC system with flow rates of 0.8ml/minute and 1.2ml/minute wavelength of (±5nm).

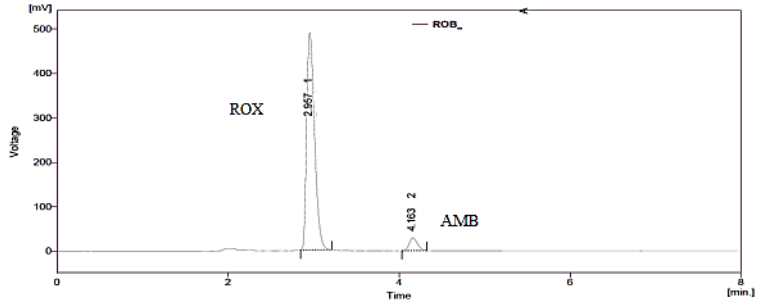

Figure.7.Chromatogram for robustness at change in buffer pH

Table.9.Showing validation report

Parameter	Requirement	Results		Acceptance criteria
		Roxithromycin	Ambroxol hydrochloride	
Specificity	No interference	Pass	Pass	No interference
Linearity	Correlation coefficient	0.997	0.998	NLT 0.999
Accuracy	% recovery	99.52%	99.53%	100 ± 2.0%
Method Precision	% RSD	0.53	0.40	NMT 2%
	% RSD	0.65	0.34	NMT 2%
System precision Ruggedness	% RSD	0.38	0.49	NMT 1%
Robustness	% RSD	0.43	0.36	NMT 1%
System suitability	RT	2.837	4.023	-
	Tailing factor	1.6	1.3	NMT 2
	Plate count	4468	8129	NLT 3000
	Assay value	99.47%	99.98%	100 ± 2.0%

4. CONCLUSION

The HPLC method was found to be accurate, precise, economical and reproducible. The method can be suggested for routine analysis and method can be recommended for determination of substance related, relative substance of Roxithromycin and Ambroxol hydrochloride in AIP and combined pharmaceutical dosage form.

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