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High Performance Liquid Chromatographic Determination of Ambroxol, Roxithromycin and Serratiopeptidase in Combined Tablet Dosage Form

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A simple, sensitive and accurate RP-HPLC method for the simultaneous quantification of ambroxol, roxithromycin and serratiopeptidase in bulk and in combined tablet dosage form was developed and validated. Ambroxol, roxithromycin and serratiopeptidase were separated and estimated using Waters HPLC system and YMC Pack pro C18 (250 mm × 4.6 mm, 5 µm particle size) column. The mobile phase used was 0.1 % orthophosphoric acid and acetonitrile in the ratio of 60:40 (v/v). The calibration curves were linear over concentration range of 12-36, 60-180 and 6-18 µg/mL with limits of detection of 0.040, 1.176 and 0.127 µg/mL for ambroxol, roxithromycin and serratiopeptidase, respectively. For the studied drugs, recovery varied in range of 99.57 to 100.24 % with relative standard deviation ranging from 0.12 to 0.36 %. The proposed RP-HPLC method could be used for the analysis of ambroxol, roxithromycin and serratiopeptidase simultaneously in combined tablet dosage forms.

Keywords: Ambroxol, Roxithromycin, Serratiopeptidase, HPLC analysis, Tablets.

INTRODUCTION

Ambroxol (AX) [1-3] is a potent mucolytic agent. Chemically, it is known as 4-[(2-amino-3,5-dibromophenyl)methylamino]cyclohexan-1-ol. It is used in the treatment of variety of respiratory disorders such as emphysema with bronchitis pneumoconiosis, chronic inflammatory pulmonary conditions, tracheobronchitis, bronchiectasis, bronchitis with bronchospasm asthma. It exerts its mucolytic activity by facilitating the breakdown of acid mucopolysaccharide fibres in the mucous and thus making it thinner and less viscous. Therefore, easy for expectoration of the mucus.

Roxithromycin (RM) [4-6], a semi-synthetic antibiotic, belongs to macrolides class of organic compounds. It is used in the treatment of respiratory tract infections, pneumonia and can be used for skin infections caused by bacteria. Roxithromycin prevents bacterial growth, by binding irreversibly to the 50S subunit of the bacterial ribosome and thus, interfering the translocation step in bacterial protein synthesis.

Serratiopeptidase (SPD) [7,8] is a proteolytic enzyme produced by *Serratia marcescens* through aerobic fermentation processes. Serratiopeptidase possesses anti-inflammatory properties and is more effective compared to non-steroidal anti-inflammatory agents. Topical formulations of serratiopeptidase would be useful to treat local inflammations.

The literature survey reveals that no analytical methods were reported for the simultaneous estimation of ambroxol, roxithromycin and serratiopeptidase in bulk and combined dosage form. Therefore, the objective of the present study is to develop a novel analytical method for the simultaneous analysis of ambroxol, roxithromycin and serratiopeptidase by employing RP-HPLC with photodiode array detector (PDA). The validation of the developed method was performed according to ICH guidelines [9].

EXPERIMENTAL

Ambroxol, roxithromycin and serratiopeptidase samples were gifted by Lara Drugs Private Limited (Telangana, India). Orthophosphoric acid (Merck Pvt. Ltd., Mumbai, India) and acetonitrile (Merck Pvt. Ltd., Mumbai, India) of HPLC grade were used during the present study. Purified water was obtained from a Milli-Q system. Amrox-S tablets, labeled to contain ambroxol - 30 mg, roxithromycin - 150 mg and SDP - 15 mg (Norwest Pharmaceuticals Inc. New Delhi, India), were purchased from the pharmacy market. Waters HPLC system consisting of a binary HPLC pump model 2695, PDA detector model 2998, vacuum degasser and Waters Empower2 software;

and YMC Pack pro C18 (250 mm × 4.6 mm, 5 µm particle size) analytical column were used in this study.

Chromatographic conditions: Separation of ambroxol, roxithromycin and serratiopeptidase was achieved using YMC Pack pro C18 (250 mm × 4.6 mm, 5 µm particle size) analytical column as the stationary phase and 0.1 % orthophosphoric acid and acetonitrile in the ratio of 60:40 (v/v) as mobile phase. The mobile phase was filtered by passing through a membrane filter prior to use. Isocratic elution was achieved at a flow rate of 1.0 mL/min with a column temperature of 30 °C. The injection volume was 10 µL. The chromatograms were recorded at 290 nm using PDA detector.

Stock and working standard solutions: The mobile phase was used as the diluent. Accurately weighed 30 mg of ambroxol, 150 mg of roxithromycin and 15 mg of serratiopeptidase were transferred into a 100 mL volumetric flask, 30 mL of mobile phase was added and the mixture was sonicated to dissolve it. The resulting mixture was made upto the volume with the same solvent. Working standard solution was prepared by diluting above stock solution using the mobile phase to produce 24, 120 and 12 µg/mL of ambroxol, roxithromycin and serratiopeptidase, respectively.

General assay procedure: The working standard solutions were prepared in the concentration range of 12-36, 60-180 and 6-18 µg/mL for ambroxol, roxithromycin and serratiopeptidase, respectively by dilution of the stock solution using the diluent. These were injected in triplicate. The chromatograms were recorded under the earlier described chromatographic conditions. The peak areas were plotted against the corresponding concentrations to construct the calibration curve.

Assay of tablet dosage form: For the determination of ambroxol, roxithromycin and serratiopeptidase in combined tablet dosage form, 20 Amrox-S tablets were weighed and finely powdered. Suitable portion of powder equivalent to 30 mg of ambroxol, 150 mg of roxithromycin and 15 mg of serratiopeptidase was accurately weighed and transferred to a 100 mL volumetric flask. The flask was made up to volume with diluent and sonicated for 15 min. The solution was then passed through 0.45 µm membrane filter and diluted to produce 24 µg/mL (ambroxol), 120 µg/mL (roxithromycin) and 12 µg/mL (serratiopeptidase). The sample solution was treated same as the general assay procedure. Recovered concentrations of ambroxol, roxithromycin and serratiopeptidase were calculated from the corresponding calibration curves.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions: The most important phase in the RP-HPLC method development is the achievement of adequate resolution with acceptable peak symmetry in a reasonable analysis time. To accomplish this, several experiments were carried out so as to optimize the stationary phase and mobile phase. For the stationary phase, two analytical columns Zorbax C18 (250 mm × 4.6 mm, 5 µm particle size) and YMC Pack pro C18 (250 mm × 4.6 mm, 5 µm particle size) were tested. Successful resolution and acceptable peak symmetry of the drugs were attained by using the Zorbax C18 (250 mm × 4.6 mm, 5 µm particle size) analytical column. Hence used in the present study.

Different mobile phase combinations using different proportions and flow rates such as 0.1 M NaH_2PO_4 with acetonitrile, 0.1 M ammonium acetate with acetonitrile and 0.1 % orthophosphoric acid with acetonitrile were tested. The best resolution of the three drugs within acceptable analysis time was obtained through an isocratic elution using a mobile phase consisting of 0.1 % orthophosporic acid and acetonitrile in the ratio 60:40 (v/v) at a flow rate of 1 mL/min. Quantification was done using PDA. The three drugs exhibited considerable absorption at 290 nm, hence selected for quantification.

The above described chromatographic conditions showed symmetric peaks and sufficient resolution between ambroxol, roxithromycin and serratiopeptidase. Fig. 1 shows a typical chromatogram for the separation of three drugs. Ambroxol, roxithromycin and serratiopeptidase eluted at retention times 2.494, 4.382 and 11.934 min, respectively.

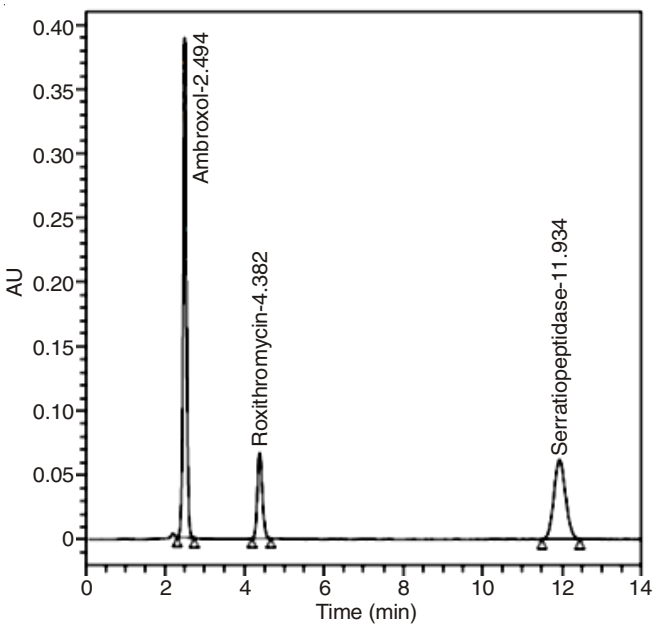

Fig. 1. Chromatogram of ambroxol, roxithromycin and serratiopeptidase

System suitability test: The suitability of the developed method was verified by five replicates of working standard solution (24 µg/mL ambroxol, 120 µg/mL roxithromycin and 12 µg/mL serratiopeptidase). The USP plate count, USP tailing factor, USP resolution and repeatability of the retention time and peak areas were determined. The results and the acceptable limits are shown in Table-1.

Linearity and concentration ranges: The linearity of the proposed HPLC method was evaluated by analyzing a series of different concentrations (n = 5) for each of the three drugs. The linear regression equations were generated by least square method of the calibration data. Under the optimized chromatographic conditions, the measured peak areas of ambroxol, roxithromycin and serratiopeptidase at 290 nm were found to be proportional to their concentrations. Table-2 presents the linear regression equations, concentration ranges, regression coefficients, intercept and slope. Regression analysis shows good linearity as indicated from the correlation coefficient values (≥ 0.9990).

TABLE-1
SYSTEM SUITABILITY PARAMETERS

Parameters	Results			Recommended limits
	Ambroxol	Roxithromycin	Serratiopeptidase	
Retention time	2.494 (% RSD - 0.29)	4.382 (% RSD - 0.14)	11.934 (% RSD - 0.33)	RSD ≤ 1
Peak area	2236988 (% RSD - 0.31)	574832 (% RSD - 0.12)	1238416 (% RSD - 0.36)	RSD ≤ 1
USP resolution	–	9.74	19.42	> 1.5
USP plate count	4344	5969	7873	> 2000
USP tailing factor	1.07	1.15	1.07	≤ 2

TABLE-2
LINEARITY, REGRESSION AND SENSITIVITY PARAMETERS FOR THE DETERMINATION OF AMBROXOL, ROXITHROMYCIN AND SERRATIOPEPTIDASE

Parameter	Ambroxol	Roxithromycin	Serratiopeptidase
Linearity range (µg/mL)	12-36	60-180	6-18
Regression equation: ($y^a = m\ x^b + c$)	y = 93382x + 8830	y = 4906x – 1315	y = 10497x + 416.4
Slope (m)	93382	4906	10497
Intercept (c)	8830	-1315	416.4
Regression coefficient (R^2)	0.9999	0.9990	0.9999
Limit of detection (µg/mL)	0.040	1.176	0.127
Limit of quantification (µg/mL)	0.134	3.922	0.423

[a]Peak area of the drug: [b]Concentration of drug in µg/mL

Sensitivity: Sensitivity of the method was assessed by determining limit of detection (LOD) and limit of quantification (LOQ). Limit of detection and limit of quantification were calculated as signal-to-noise ratio of 3:1 and 10:1, respectively. The limit of detection and limit of quantification values for ambroxol, roxithromycin and serratiopeptidase were calculated and are presented in Table-2.

Precision: The precision for the proposed method was studied at a concentration of 24 µg/mL ambroxol, 120 µg/mL roxithromycin and 12 µg/mL serratiopeptidase using six replicate determinations. The percentage relative standard deviation (% RSD) did not exceed 1 % proving the high repeatability of the developed method (Table-3).

TABLE-3
RESULTS OF PRECISION OF THE METHOD

Ambroxol		Roxithromycin		Serratiopeptidase	
Peak area	RSD (%)	Peak area	RSD (%)	Peak area	RSD (%)
2265429	0.31	593565	0.12	1273586	0.36
2273949		592623		1269361	
2256539		592266		1268952	
2269736		591966		1259731	
2256419		592793		1268201	
2266289		593738		1266138	

Accuracy: The accuracy of the proposed method was established by means of the standard addition technique, by adding a known amount of standard drug at three different levels (50, 100 and 150 %) to the preanalyzed sample. Accuracy was expressed as percentage recovery in Table-4. The accuracy of the developed method for the ambroxol, roxithromycin and serratiopeptidase ranged from 99.57 to 100.24 % indicating acceptable accuracy.

Robustness: The robustness of the developed method was checked by studying the effect of deliberate changes in the flow rate of mobile phase (± 0.1 mL/min) and column temperature (± 2 °C) on the chromatographic system suitability parameters.

TABLE-4
RESULTS OF ACCURACY OF THE METHOD

Spiked level (%)	Concentration of drug (µg/mL)		Recovery (%)	Mean (%)
	Added	Found		
Ambroxol				
50	12.00	11.97	99.75	99.83
	12.00	11.96	99.66	
	12.00	12.01	100.08	
100	24.00	24.02	100.08	100.07
	24.00	23.93	99.71	
	24.00	24.10	100.42	
150	36.00	35.94	99.83	99.73
	36.00	35.88	99.66	
	36.00	35.90	99.72	
Roxithromycin				
50	60.00	59.90	99.83	100.01
	60.00	60.11	100.18	
	60.00	60.01	100.02	
100	120.00	120.71	100.59	100.24
	120.00	119.98	99.98	
	120.00	120.18	100.15	
150	180.00	179.44	99.68	99.73
	180.00	179.81	99.89	
	180.00	179.34	99.63	
Serratiopeptidase				
50	6.00	6.00	100.00	100.22
	6.00	6.01	100.16	
	6.00	6.03	100.50	
100	12.00	12.04	100.33	100.08
	12.00	12.00	100.00	
	12.00	11.99	99.91	
150	18.00	17.95	99.72	99.57
	18.00	17.92	99.55	
	18.00	17.90	99.44	

According to the results shown in Table-5, these small and deliberate variations did not have any significant effect on the measured system suitability parameters.

TABLE-5
INFLUENCE OF SMALL CHANGES IN FLOW RATE AND COLUMN TEMPERATURE (METHOD ROBUSTNESS)

Parameter	Value	Retention time	Peak area	USP plate count	USP tailing
		Roxithromycin			
Temperature (°C)	30 – 1	3.412	3225247	5368	1.18
	30 +1	2.684	2446899	4695	1.17
Flow rate (mL/min)	1 – 0.1	3.404	3221243	5217	1.18
	1 + 0.1	2.686	2517844	4650	1.16
		Ambroxol			
Temperature (°C)	30 – 1	3.412	3225247	5368	1.10
	30 + 1	2.684	2446899	4695	1.07
Flow rate (mL/min)	1 – 0.1	3.404	3221243	5217	1.11
	1 + 0.1	2.686	2517844	4650	1.09
		Serratiopeptidase			
Temperature (°C)	30 – 1	16.321	1807276	9201	1.08
	30 + 1	13.073	1358849	8478	1.06
Flow rate (mL/min)	1 – 0.1	16.329	1765474	9379	1.07
	1 + 0.1	12.819	1374136	8045	1.08

Conclusion

The present study describes a simple and reliable isocratic RP-HPLC with PDA detection procedure for the assay of ambroxol, roxithromycin and serratiopeptidase simultaneously in bulk and in their combined pharmaceutical dosage form. The developed method was validated as per the ICH guidelines. The results of validation showed that the developed method proved to be linear, sensitive, precise, accurate, robust and convenient. Hence, the developed method can be used for routine quality control analysis purpose.

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