Development and Validation of RP-HPLC Method for Simultaneous Estimation of Bromhexine and Erythromycin in Bulk and Pharmaceutical Dosage Forms

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Abstract

A simple, rapid, accurate and precise isocratic reversed phase high performance liquid chromatographic method has been developed and validated for simultaneous estimation of Bromhexine and Erythromycin in tablet dosage forms. The chromatographic separation was carried out on Hypersil BDS C18 column (150 mm x 4.6 mm I.D., 5 µm particle size) with a mixture of 0.01N phosphate buffer and acetonitrile in the ratio of 35:65 v/v as a mobile phase at a flow rate of 1.0 mL/min. UV detection was performed at 224 nm. The retention times were 4.619 minutes and 2.329 minutes for Bromhexine and Erythromycin respectively. Calibration plots were linear (r^2=0.999 for both Bromhexine and Erythromycin respectively) over the concentration range of 2-12 µg/mL for Bromhexine and 62.5-375 µg/mL for Erythromycin. The method was validated for linearity, precision, accuracy and robustness. The proposed method was successfully used for simultaneous estimation of Bromhexine and Erythromycin in tablet dosage forms. Validation studies revealed that the proposed method is specific, rapid, reliable and reproducible. The high % recovery and low % RSD values confirm the suitability of the proposed method for routine quality control analysis of Bromhexine and Erythromycin in bulk and tablet dosage forms.

Keywords: Bromhexine, Erythromycin, Estimation, HPLC

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Introduction

Bromhexine is a mucolytic (expectorant) agent used in the treatment of respiratory disorders associated with viscid or excessive mucus[1]. Bromhexine is intended to support the body's mechanisms for clearing mucus from the respiratory tract. Chemically it is, 2,4-dibromo-6-{[cyclohexyl(methyl)amino]methyl} aniline[2] (Fig. 1). Bromhexine acts on the mucus at the formative stages in the glands, within the mucus-secreting cells. Bromhexine disrupts the structure of acid mucopolysaccharide fibres in mucoid sputum and produces less viscous mucus, which is easier to expectorate[3].

Erythromycin is a macrolide antibiotic produced by *Streptomyces erythraea*. Erythromycin is an antibacterial agent and used to treat or prevent many different types of infections caused by bacteria[4]. Chemically it is, (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-6-{[(2*S*,3*R*,4*S*,6*R*)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-14-ethyl-7,12,13-trihydroxy-4-{[(2*R*,4*R*,5*S*,6*S*)-5-hydroxy-4-methoxy-4,6-dimethyl oxan-2-yl]oxy}-3,5,7,9,11,13-hexamethyl-1-oxacyclo tetradecane-2,10-dione (Fig. 2). Erythromycin inhibits bacterial protein synthesis by binding to bacterial 50S ribosomal subunits; binding inhibits peptidyltransferase activity and interferes with translocation of amino acids during translation and assembly of proteins[5].

Literature survey reveals that there is no HPLC method was reported for simultaneous estimation of Bromhexine and Erythromycin in pharmaceutical formulations. Therefore, an attempt has been made to develop a novel, rapid, accurate and precise RP-HPLC method for simultaneous estimation of Bromhexine and Erythromycin in tablet dosage form and validated in accordance with ICH guidelines[6].

Materials and Methods

Instrumentation: To develop a high performance liquid chromatographic method for simultaneous estimation of Bromhexine and Erythromycin using Waters 2695 HPLC system on Hypersil BDS C18 (150 mm x 4.6 mm I.D., 5 µm particle size) column was used. The instrument is equipped with an auto sampler and UV-Vis detector. A 20 µL rheodyne injector port was used for injecting the samples. Data was analyzed by using Empower 2 software. A Systronics-361 pH meter was used for pH measurements.

Chemicals and solvents: The working standards of Bromhexine Hydrochloride and Erythromycin Stearate were provided as gift samples from Spectrum Pharma Research Solutions, Hyderabad, India. The marketed formulation of Bromhexine and Erythromycin tablets (Bromhexine Hydrochloride 8 mg and Erythromycin Stearate 250 mg) were procured from local market. HPLC grade water and acetonitrile were purchased from E. Merck (India) Ltd., Mumbai, India. Methanol and

potassium dihydrogen phosphate of AR grade was obtained from S.D. Fine Chemicals Ltd., Mumbai, India.

Chromatographic conditions: 0.01N Phosphate buffer and acetonitrile in the ratio of 35:65 v/v was found to be the most suitable mobile phase for ideal chromatographic separation for simultaneous estimation of Bromhexine and Erythromycin. The solvent mixture was filtered through 0.45 µm membrane filter and sonicated before use. It was pumped through the column at a flow rate of 1.0 mL/min. Injection volume was 10 µL and the column was maintained at a temperature of 30^0C. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solution. The detection of the drug was monitored at 224 nm. The run time was set as 7 minutes.

Preparation of 0.01N phosphate buffer: 1.36 grams of potassium dihydrogen phosphate was weighed accurately, transferred into a 1000 mL volumetric flask and dissolved in 500 mL of HPLC grade water. The solution was sonicated for 30 minutes, degassed and then made to total volume with water. The resulting solution was filtered through 0.45 µm membrane filter.

Preparation of mobile phase and diluent: The mobile phase was prepared by mixing 350 mL of 0.01N phosphate buffer with 650 ml of acetonitrile. The solution was degassed in an ultrasonic water bath for 5 minutes and filtered through 0.45 µm filter under vacuum. The solution of 500 mL of water and 500 mL of acetonitrile was used as diluent.

Preparation of standard solution: Accurately weighed and transferred 8 mg of Bromhexine and 250 mg of Erythromycin working standards into 100 mL and 25 mL volumetric flasks and was dissolved in $3/4^{th}$ volume of diluent. Sonicated the solution for few minutes and dissolved the drugs completely and make up to the final volume with diluent. Then it was filtered through 0.45 µm filter. From this stock solutions 1 mL was transferred into 10 mL volumetric flasks and diluted up to the mark with diluent.

Preparation of sample preparation: Twenty commercial tablets were weighed, powdered and weighed accurately the tablet powder equivalent to 8 mg of Bromhexine and 250 mg of Erythromycin, transferred in to 100 mL volumetric flask and was dissolved in 70 mL of the diluent. Sonicated the solution for few minutes and dissolved the drugs completely and make up to the final volume with diluent. Then it was filtered through 0.45 µm filter. From this stock solution 1 mL was transferred into 10 mL volumetric flask and diluted up to the mark with diluent.

Procedure

The column was maintained at a temperature of 30^0C. The run time was set at 7 minutes. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solutions. Inject 10 µL of the standard and sample solutions six times into the chromatographic system at a flow rate of 1.0 mL/min and the corresponding chromatograms were obtained. From these chromatograms, the average area under the peak of each dilution was computed.

Method validation

Linearity: Several aliquots of standard solutions of Bromhexine and Erythromycin were taken in six different 10 mL volumetric flasks and diluted up to the mark with diluent such that the final concentrations were in the range of 2-12 µg/mL for Bromhexine and 62.5-375 µg/mL for Erythromycin. The above solutions were injected into the HPLC system keeping the injection volume constant. The drugs were eluted with UV detector at 224 nm, peak areas was recorded for all the peaks. The linearity curves were constructed by plotting concentration of the drugs against peak areas. The regression equation of this curve was computed. This regression equation was later used to estimate the amount of drugs in tablet dosage forms.

Precision: Precision for Bromhexine and Erythromycin was determined in terms of intra-day precision and inter-day precision. Every sample was injected six times. The measurements for peak areas were expressed in terms of % RSD.

Accuracy: The accuracy of the method was assessed by recovery studies of Bromhexine and Erythromycin at three concentration levels 50%, 100% and 150%. Fixed amount of pre-analyzed sample was spiked with known amount of Bromhexine and Erythromycin. Each level was repeated three times. The % recovery of Bromhexine and Erythromycin were calculated.

System suitability: The system suitability parameters like retention time, theoretical plates and tailing factor were evaluated by six replicate analysis of Bromhexine and Erythromycin and compared with standard values. The acceptance criteria are % RSD of peak areas not more than 2%, theoretical plates numbers (N) at least 3000 per each peak and tailing factors not more than 2.0 for Bromhexine and Erythromycin.

Limit of detection and limit of quantification: The limit of detection (LOD) and limit of quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions of Bromhexine and Erythromycin using the developed HPLC method. LOD and LOQ were estimated from signal-to-noise ratio. LOD and LOQ

were calculated using 3.3 σ/s and 10 σ/s formulae, respectively. Where, σ is the standard deviation of the peak areas and S is the slope of the corresponding calibration curve.

Robustness: The robustness of the method was determined by making small deliberate changes in method like variation of flow rate, mobile phase ratio and temperature.

Assay: Standard preparations are made from the bulk drug and sample preparations are made from formulation. Both standard and sample solutions were injected in six homogeneous samples. 10 µL of sample solution was injected and from the peak areas of Bromhexine and Erythromycin, amount of each drug in the sample were computed. The results were compared with the label claim of Bromhexine and Erythromycin in tablet dosage forms. From the results the average % Assay was calculated.

Results and Discussion

The HPLC procedure was optimized with a view to develop an accurate, precise and reproducible method for simultaneous estimation of Bromhexine and Erythromycin in tablet dosage form using Hypersil BDS C18 column (150 mm x 4.6 mm; 5 µm) in isocratic mode with mobile phase composition of 0.01N phosphate buffer and acetonitrile in the ratio 35:65 v/v. The use of 0.01N phosphate buffer and acetonitrile in the ratio of 35:65 v/v resulted in peak with maximum separation, good shape and resolution. Flow rates between 0.8 to 1.2 mL/min were studied. A flow rate of 1.0 mL/min gave an optimum signal-to-noise ratio with reasonable separation time, the retention times for Bromhexine and Erythromycin were found to be 4.619 minutes and 2.329 minutes respectively. Total run time was 7 minutes. The drug components were measured with UV detector at 224 nm. The results of optimized chromatographic conditions were shown in Table 1.

Table 1: Optimized chromatographic conditions

S. No.	Parameter	Condition
1	Mobile phase	Phosphate buffer: acetonitrile (35:65 v/v)
2	Diluent	Water: acetonitrile (50:50 v/v)
3	Column	Hypersil BDS C18 (150 mm x 4.6 mm, 5 µm)
4	Column temperature	30^0C
5	Wave length	224 nm
6	Injection volume	10 µL
7	Flow rate	1.0 mL/min.
8	Run time	7 min.

Linearity was obtained in the range of 2-12 µg/mL for Bromhexine and 62.5-375 µg/mL for Erythromycin. The correlation coefficient (r^2) was found to be 0.999 for both Bromhexine and Erythromycin respectively. The regression equation of the linearity plot of concentration of Bromhexine over its peak area was found to be y=68728x+1698, where x is the concentration of Bromhexine (µg/mL) and y is the corresponding peak area. The regression equation of the linearity plot of concentration of Erythromycin over its peak area was found to be y=4065x+553, where x is the concentration of Erythromycin (µg/mL) and y is the corresponding peak area.

The results show that an excellent correlation exists between peak area and concentration of drugs within the concentration range indicated. The linearity results were shown in Table 2 and Table 3 and the calibration curves were shown in Fig. 3 and Fig. 4.

Table 2: Linearity results of Bromhexine

S. No.	Concentration (µg/mL)	Mean peak area
1	2	128445
2	4	287816
3	6	422905
4	8	553028
5	10	678397
6	12	827890

Table 3: Linearity results of Erythromycin

S. No.	Concentration (µg/mL)	Mean peak area
1	62.5	253537
2	125	498875
3	187.5	773594
4	250	1011472
5	312.5	1290713
6	375	1504001

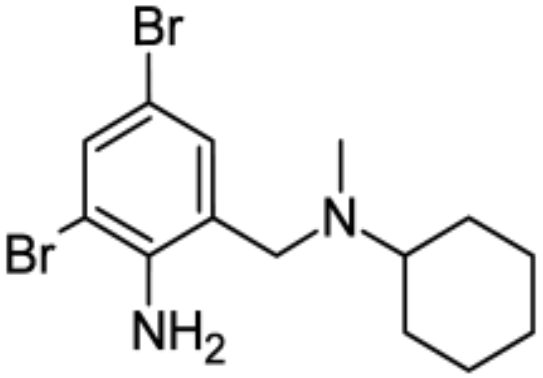


Fig. 1: Molecular structure of Bromhexine

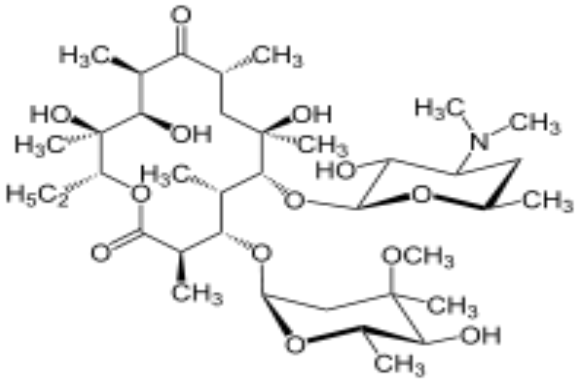


Fig. 2: Molecular structure of Erythromycin

Fig. 3: Calibration curve of Bromhexine

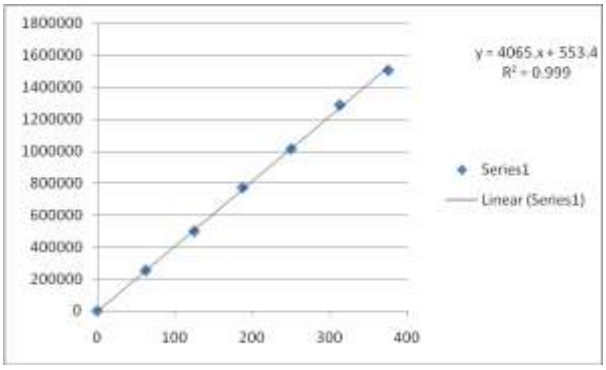
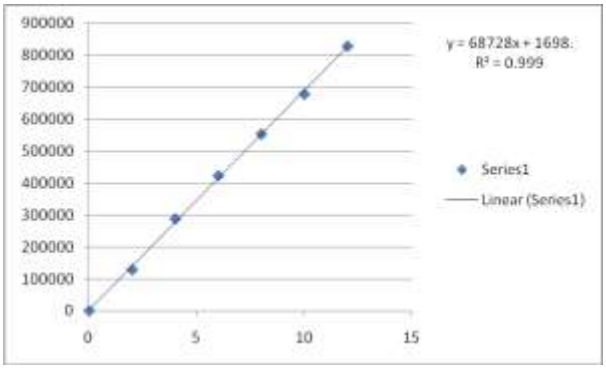


Fig. 4: Calibration curve of Erythromycin



The % RSD for intra-day precision and inter-day precision for Bromhexine were found to be 0.24% and 0.74% respectively (limit % RSD<2.0%). The % RSD for intra-day precision and inter-day precision for Erythromycin were found to be 0.10% and 0.22% respectively (limit % RSD<2.0%) and hence the method is precise. The precision data of Bromhexine and Erythromycin were furnished in Table 4 and Table 5.

Table 4: Precision data of Bromhexine

S. No.	Peak area	
	Intra-day precision	Inter-day precision
Injection-1	586255	576392
Injection-2	585751	570523
Injection-3	589801	572766
Injection-4	588027	570336
Injection-5	587096	579984
Injection-6	587375	578999
Mean	587375	574833
SD	1434.1	4228.2
% RSD	0.24	0.74

Table 5: Precision data of Erythromycin

S. No.	Peak area	
	Intra-day precision	Inter-day precision
Injection-1	1016642	1035747
Injection-2	1016742	1030362
Injection-3	1015539	1035837
Injection-4	1018073	1032412
Injection-5	1015565	1031710
Injection-6	1016878	1032170
Mean	1016573	1033040
SD	945	2246.9
% RSD	0.1	0.22

The % recovery of the drugs Bromhexine and Erythromycin were found to be 100.25 to 100.43% and 100.58 to 102.20% respectively and the high percentage of recovery of Bromhexine and Erythromycin indicates that the proposed method is highly accurate. The results of accuracy studies of Bromhexine and Erythromycin were shown in Table 6 and Table 7.

Table 6: Accuracy studies of Bromhexine

% Concentration level	Conc. added (µg/mL)	Conc. found (µg/mL)	% Recovery
50%	4	4.017	100.42%
100%	8	8.035	100.43%
150%	12	12.03	100.25%

Table 7: Accuracy studies of Erythromycin

% Concentration level	Conc. added (µg/mL)	Conc. found (µg/mL)	% Recovery
50 %	125	125.73	100.58%
100%	250	253.75	101.50%
150%	375	383.25	102.20%

The retention times for the drugs Bromhexine and Erythromycin was 4.619 minutes and 2.329 minutes respectively. The number of theoretical plates calculated for Bromhexine and Erythromycin was 5751 and 4606 respectively. The tailing factor for Bromhexine and Erythromycin was 1.84 and 1.31 respectively, which indicates efficient performance of the column. The limit of detection (LOD) and limit of quantification (LOQ) for Bromhexine were found to be 0.018 µg/mL and 0.055 µg/mL; 0.160 µg/mL and 0.485 µg/mL for Erythromycin respectively, which indicate the sensitivity of the method. The summary of system suitability parameters and validation parameters were shown in Table 8.

The robustness studies indicated that no considerable effect on the determination of the drugs.

Table 8: System suitability parameters of proposed method

S. No.	Parameters	Bromhexine	Erythromycin
1	Linearity (µg/mL)	2-12	62.5-375
2	Correlation coefficient	0.999	0.999
3	Retention time (min.)	4.619	2.329
4	Resolution	11.69	---
5	Tailing factor	1.84	1.31
6	Theoretical plates (N)	5751	4606
7	LOD (µg/mL)	0.018	0.160
8	LOQ (µg/mL)	0.055	0.485

Therefore the test method is robust for the quantification of the drugs. In all deliberately varied conditions, the % RSD for replicate injections of Bromhexine and Erythromycin were found to be within the acceptable limits.

Validated method was applied for the simultaneous estimation of Bromhexine and Erythromycin in commercial tablet dosage forms. The % Assay of Bromhexine and Erythromycin were found to be 100.86% and 101.50% respectively. The results for the drugs assay showed good agreement with label claims. No interfering peaks were found in the chromatogram of the tablet formulation within the run time indicating that excipients used in tablet formulation did not interfere with the simultaneous estimation of the drugs Bromhexine and Erythromycin by the proposed HPLC method. The assay results are shown in Table 9.

Table 9: Assay results of marketed formulations

S. No.	Bromhexine % Assay	Erythromycin % Assay
1	100.40	101.78
2	99.38	101.26
3	99.77	101.79
4	99.35	101.46
5	101.03	101.39
6	100.86	101.43
Mean	100.86	101.50
SD	0.7365	0.2208
% RSD	0.74	0.22

The chromatograms were checked for appearance of any extra peaks under optimized conditions, showing no interference from common tablet excipients and impurities. Also the peak areas were compared with standard and were found to be within limits. As shown in chromatogram, two analytes are eluted by forming symmetrical peaks. The typical chromatogram of

Bromhexine and Erythromycin standard were shown in Fig. 5.

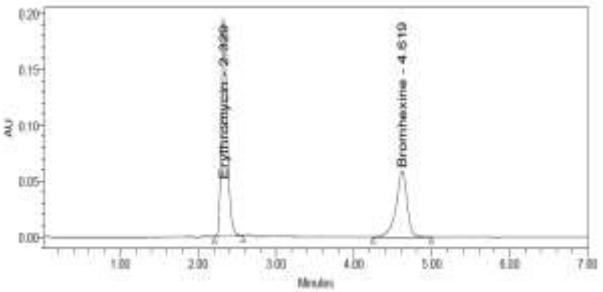


Fig. 5: Typical chromatogram of standard for Bromhexine and Erythromycin

Conclusion

The proposed HPLC method is rapid, sensitive, precise and accurate for the simultaneous estimation of Bromhexine and Erythromycin and can be reliably adopted for routine quality control analysis of Bromhexine and Erythromycin in its tablet dosage forms.

Source of Support: None

Conflict of Interest: Nil

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