

Development and Validation of Analytical Method for Simultaneous Estimation of Bromhexine HCl and Enrofloxacin in Combined Pharmaceutical Dosage Form

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Received 13 June 2017 • Revised 25 August 2017 • Accepted 5 October 2017

ABSTRACT

A simple, accurate, precise RP-HPLC method has been developed and validated for the simultaneous estimation of Bromhexine HCl and Enrofloxacin in their combined tablet dosage form. The combination used for the Separation is ENROLIQ-B. Separation was performed on a C₁₈ column [Hypersil BDS C₁₈ column, 250 x 4.6 mm], with 0.05 M KH₂PO₄ Buffer pH 6: Methanol: Triethylamine (70:30:0.1 %v/v/v) as a mobile phase and flow rate was kept at 1 ml/min. Good sensitivity was found with UV detection at 261.00 nm. After method development the interference with the active compounds and excipients, repeatability and linearity were investigated. Retention times were found to be 3.190 and 5.067 min. respectively, for BROM and ENR. The method was validated over the analytical range from 1.5-4.5 µg/ml for BROM (r²=0.998) and 20-60 µg/ml for ENR (r²=0.999). This method showed good reproducibility and recovery with %RSD in the desired range. The proposed method can be successfully applied for the routine analysis of both drugs in their combine dosage form.

Keywords: Bromhexine HCl (BROM), Enrofloxacin (ENR) Methanol, RP-HPLC method, validation

INTRODUCTION

Bromhexine HCl (BROM) is a mucolytic agent used in the treatment of respiratory disorders marketed in combination with terbutaline (TB), a β₂-adrenergic receptor agonist used as a fast-acting bronchodilator [1] chemically known as 2-amino-3,5-dibromobenzyl (cyclohexyl) methylamine hydrochloride [2]. It is mainly used in the human in the treatment of non-productive cough. Enrofloxacin (ENR) is action. It is effective against a broad spectrum of Gram-negative bacteria and is indicated for infections of the respiratory, gastrointestinal and urinary tracts in cattle, pigs and poultry. Enrofloxacin is bactericidal through the inhibition of DNA-gyrase [3] chemically known as 1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolonecarboxylic acid. It is commercially available with fixed combination of BROM (200 mg) and ENR (15 mg) is available in the market as tablet formulation. The ratio in marketed formulation is 1:13.33 respectively, for BROM and ENR. BROM is official in IP [4] and BP [5] whereas ENR is official in USP [6]. Number of pharmacopoeial methods reported methods like UV, RP- HPLC, UV- HPTLC and LC-MS/MS methods have been developed for the estimation of BROM [7-13]. While Liquid Chromatography, TLC and HPLC is reported for estimation of ENR [14-17]. No single method has been reported till date for the simultaneous estimation of BROM and ENR using RP-HPLC method. The present paper describes the development and validation of RP-HPLC analytical method for simultaneous estimation of BROM and ENR in their combined pharmaceutical dosage form. The proposed method are optimized and validated as per ICH guidelines [18-20].

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Table 1. Results of System Suitability Parameters

Drug	Retention Time (min)	Area (mAU×min)	Height	Tailing factor	Theoretical plate	Resolution
Bromhexine HCl	3.190	153.309	28.701	1.300	7521	9.585
Enrofloxacin	5.067	5084.681	568.81	1.364	6922	

Reagent and Chemicals

API of Bromhexine HCl (Intas Pharmaceuticals Pvt. Ltd.) and Enrofloxacin (Torrent Pharmaceuticals Pvt. Ltd.) procured as gift samples. The table dosage form "ENROLIQ-B" having combination of BROM (200 mg) and ENR (15 mg) was procured commercially from the local market. All the chemicals used are of analytical grade.

Chromatographic Conditions

Mobile phase consisting of 0.05 M KH_2PO_4 Buffer pH 6: Methanol: Triethylamine (70:30:0.1 %v/v/v) was used in an isocratic mode. The mobile phase was initially filtered through by 0.45 μm millipore membrane filter and sonicated for 15 min. before use. The flow rate was maintained at 1ml/min and the injection volume was 20 μL . The UV detection was performed at 261 nm and the separation was achieved at room temperature.

EXPERIMENTAL

Preparation of Stock Solutions of BROM and MET

Accurately weighed quantity 15 mg of BROM and 20 mg of ENR were transferred into separate 100 ml volumetric flasks. BROM (volumetric flask A) and ENR (volumetric flask B) was dissolved by adding methanol and make up the volume up to mark with mobile phase. This will make stock solution having strength of 150 $\mu\text{g}/\text{ml}$ for BROM and 200 $\mu\text{g}/\text{ml}$ for ENR. From the volumetric flask 10ml aliquots was transferred into 100 ml vol. flask and volume was made with mobile phase to make 15 $\mu\text{g}/\text{ml}$, from which 1.0 ml was taken in 10 ml vol. flask and volume was adjusted with mobile phase to get final dilution of 1.5 $\mu\text{g}/\text{ml}$ of BROM. From volumetric flask B 1.0 ml aliquots was taken in 10 ml vol. flask and volume was adjusted with mobile phase to get final dilution of 200 $\mu\text{g}/\text{ml}$ of ENR, from which 1.0 ml was taken in 10 ml vol. flask and volume was adjusted with mobile phase to get final dilution of 20 $\mu\text{g}/\text{ml}$ of ENR.

Preparations of Sample Solutions

Accurately measure 1 ml of oral solution and transfer in to 100 ml volumetric Flask, Add about 50 ml of diluents and sonicate to dissolve it completely and mark volume up to the make with diluents. From above solution 1ml aliquots was transferred in to 10ml volumetric flask and diluted up to the mark with diluents. The solution was then filtered through 0.45 μm Millipore membrane filter. Final stock solutions containing 1.5 $\mu\text{g}/\text{ml}$ of BROM and 20 $\mu\text{g}/\text{ml}$ of ENR were prepared by subsequent dilution with mobile phase.

METHOD VALIDATION [19-20]

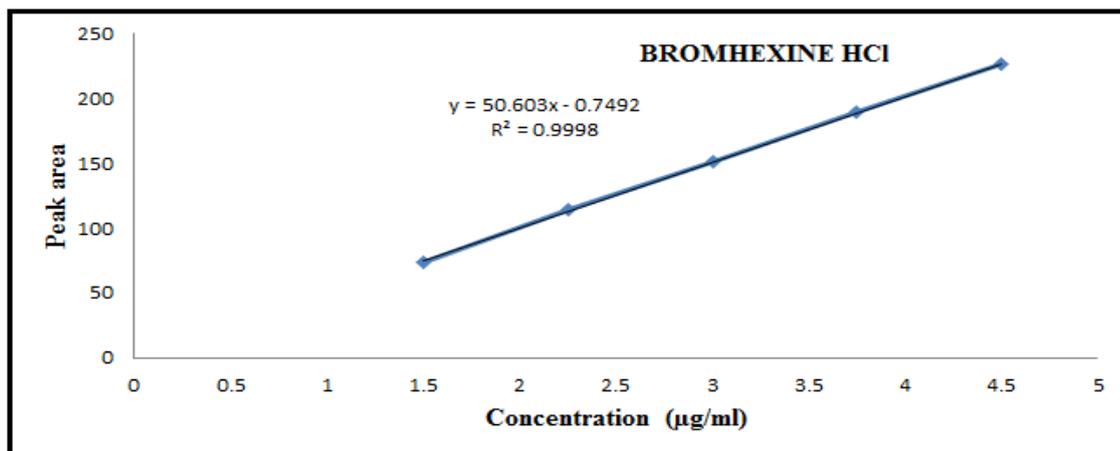
The analytical procedures were validated according to ICH Q2 B guidelines in order to determine system suitability, linearity, precision, accuracy, specificity, and robustness of analysis.

System Suitability

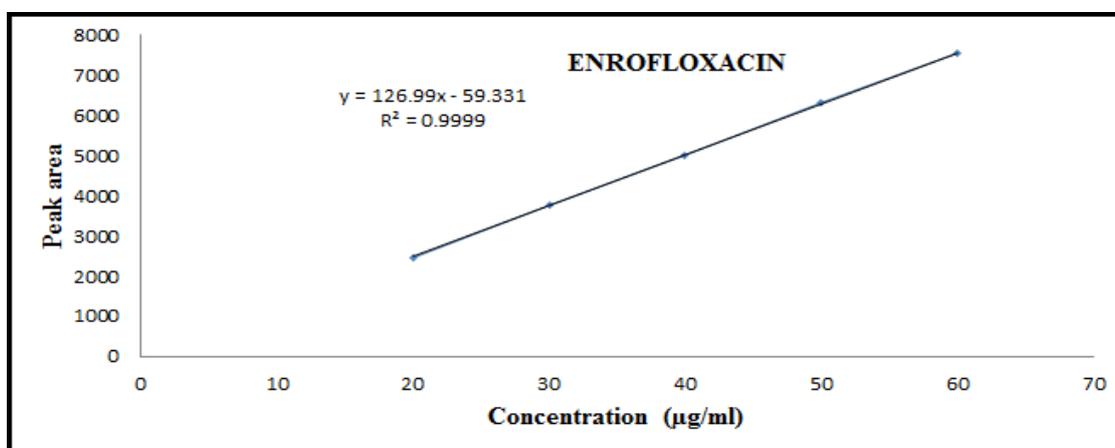
System suitability was carried out by injecting 100% concentration (sample having 1.5 $\mu\text{g}/\text{ml}$ of BROM and 20 $\mu\text{g}/\text{ml}$ of ENR) into HPLC system. This was repeated six times under the similar conditions. The tailing factor (T), number of theoretical plates (N) and resolution obtained are given in [Table 1](#).

Linearity

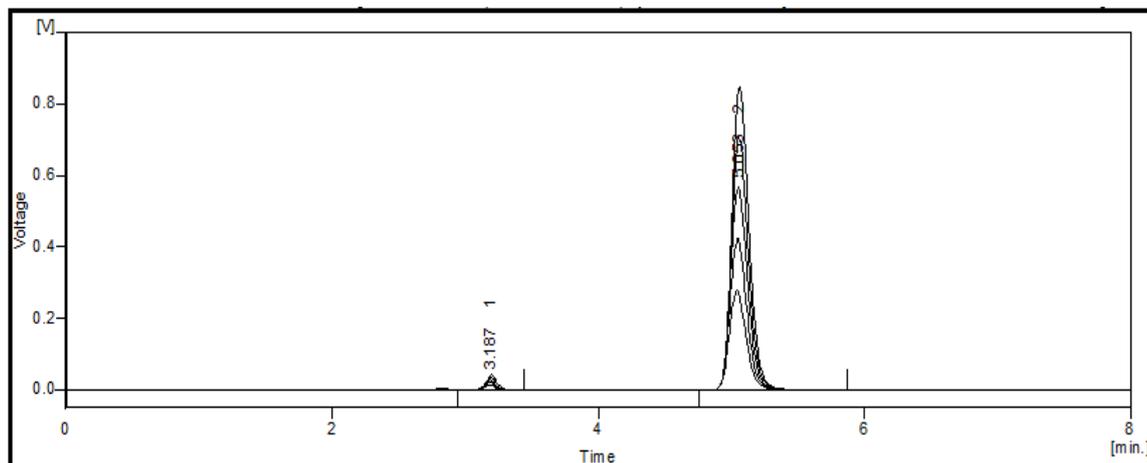
Standard calibration were prepare using five mixed standard calibration solutions in a Concentration range of 1.5-4.5 $\mu\text{g}/\text{ml}$ for BROM and 20-60 $\mu\text{g}/\text{ml}$ for ENR. Each solution was chromatographed for 08 min under the optimized chromatographic conditions. The peak areas were found out by post run analysis of the chromatogram using LC spinchrom software. Calibration curve of peak area responses versus concentration were then plotted for both drugs. The linearity of peak area responses versus concentration was demonstrated by linear least square regression analysis of the calibration curves. The calibration curves for BROM and ENR are shown in [Figure 4a](#) and



(a)



(b)



(c)

Figure 4. (a) Calibration curve of BROM; (b) Calibration curve of ENR; (c) Overlain Chromatogram for BROM and ENR

4b and their corresponding linearity parameters are given in **Table 2**. The graphs for linearity study is shown in **Figure 4a, 4b** and **4c**.

Table 2. Results of Linearity (n=6)

Regression analysis	BROM	ENR
Regression equation	$y = 50.603x - 0.7492$	$y = 126.99x - 59.331$
Correlation co-efficient(R^2)	0.9998	0.9999
Slope(S)	50.603	126.99
Intercept(6)	0.7492	59.331
LOD ($\mu\text{g/ml}$)	0.0505	0.0611
LOQ ($\mu\text{g/ml}$)	0.1533	0.1853

Table 3. Results of Precision (n=6)

Drug	Inter-day Precision (% RSD)	Intra-day Precision (%RSD)
BROM	0.49-0.68	0.33-0.68
ENR	0.36-0.96	0.23-0.59

Table 4. Results of Recovery study (Accuracy) n=3

Drug	Std. conc. Level ($\mu\text{g/ml}$)	Amount added ($\mu\text{g/ml}$)	Amt. recovered Mean \pm S.D* ($\mu\text{g/ml}$)	%Recovery
BROM	1.5	1.2	1.20 \pm 1.13	99.91
	1.5	1.5	1.50 \pm 0.72	99.51
	1.5	1.8	1.79 \pm 0.43	99.59
ENR	20	16	16.06 \pm 0.90	100.01
	20	20	19.98 \pm 0.47	99.61
	20	24	24.06 \pm 0.58	99.76

Precision

Precision was studied by measuring intra-day (repeatability which was carried out by analyzing the drug solutions on the same day) and inter-day by injecting the samples on the three consecutive days) variations of the method. Study was carried out by injecting six replicates of 100% concentration (1.5 $\mu\text{g/ml}$ of BROM and 20 $\mu\text{g/ml}$ of ENR). The % RSD values of the peak areas are given in [Table 3](#).

Accuracy

To confirm the accuracy of the proposed method, recovery experiments were performed by the standard addition technique. In this method, a known quantity of a pure drug was added at three different levels, i.e. 80%, 100% and 120% to pre-analyzed sample solutions and the recovery of BROM and MET was calculated for each concentration. The results are given in [Table 4](#).

Selectivity / Specificity

Selectivity is the ability of the analytical method to produce a response for the analyte in the presence of other interference. In order to prove that the method chosen was specific and selective. The parameters retention time (R_t), resolution (R_s) and tailing factor were calculated and given in [Table 1](#).

LOD and LOQ

LOD and LOQ of the drug were calculated using following equations according to ICH guideline $\text{LOD} = 3.3 * \sigma/S$ and $\text{LOQ} = 10 * \sigma/S$ where σ is the SD of the response and S is the slope of the calibration curve. LOD and LOQ were given in [Table 2](#).

Robustness

The Robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The Robustness was checked by changing small variation in parameters. **1)** Mobile phase flow rate ($\pm 0.2 \text{ ml/min}$) **2)** Mobile phase composition {0.05 M KH_2PO_4 Buffer pH 6: Methanol: Triethylamine (70:30:0.1 %v/v/v)} after each changes sample solution was injected and % assay with system suitability parameters were checked. The % RSD values are given in [Table 5](#).

Table 5. Results of Robustness study

Condition	Variation	BROM			ENR		
		% Assay	SD	RSD	% Assay	SD	RSD
Flow rate (1.0±0.2ml/min)	0.8 ml/min	99.66	0.60	0.581	99.89	0.67	0.649
	1.2 ml/min	98.12	0.62	0.632	98.35	0.60	0.612
Mobile phase 0.05 M KH ₂ PO ₄ pH 6 with OPA: Methanol: TEA (70:30:0.1±2 v/v/v)	72:28:0.1 (v/v/v)	99.94	0.65	0.662	99.39	0.77	0.785
	68:32:0.1 (v/v/v)	98.16	0.66	0.639	98.97	0.75	0.731
pH (6±0.2)	pH 6.2	99.41	0.60	0.588	99.91	0.47	0.457
	pH 5.8	99.65	0.69	0.724	99.96	0.77	0.804

Table 6. Results of Assay (n=3) of marketed formulation

Solution	Label Claim (mg/solution)		Amount of drug(mg)		%Assay ± SD'	
	BROM	ENR	BROM	ENR	BROM	ENR
ENROLIQ-B	15	200	14.93	199.17	99.93±0.38	98.17±9.96

RESULTS AND DISCUSSION

The objective of present work is to develop and validate RP-HPLC method which can be used for routine analysis of BROM and ENR in pharmaceutical formulation. First the chromatographic conditions were optimized for the estimation of both the drugs in selected multicomponent dosage form by RP HPLC method. A binary mixture of 0.05 M KH₂PO₄ Buffer pH 6: Methanol: Triethylamine (70:30:0.1 %v/v/v) as mobile phase was proved to be most suitable of all the combinations since the chromatographic peaks obtained were better defined and resolved and free from tailing. The detection was carried out at 261.00 nm at which both the drugs show measurable absorbance (Figure 2). The developed chromatographic method was also validated by ICH guidelines. The retention times obtained from BROM and ENR were 3.190 and 5.067 min, respectively. An optimized chromatogram showing separation of BROM and ENR at different retention time is shown in Figure 2.

System suitability was carried out by injecting 100% concentration of BROM and ENR, six times into the HPLC system. The tailing factor was less than 2 and theoretical plate number was more than 2000 for both the drugs which were within the limits. (Table 1)

The linearity and range were found to be Concentration range of 1.5-4.5 µg/ml for BROM and 20-60 µg/ml for ENR. The correlation coefficients of BROM and ENR were found to be 0.998 and 0.999, respectively for BROM and MET thus indicate good linearity in the specified concentration range (Table 2). The calibration curves for BROM and MET is shown in respectively, in Figure 4a and 4b. The minimum variation in the % RSD values obtained indicated that the present method is precise (Table 3)

The results show that the % recoveries for drug BROM and ENR were found to be 99.51-99.91% and 99.61-100.01% respectively which is well within the acceptance criteria. Hence method is can be termed accurate. Precision (Repeatability, Intraday, Interday precision -(0.1874 for BROM and 0.6359 for ENR, 0.20-0.45 for BROM and 0.20-0.35 for ENR, 0.49- 0.68 for BROM and 0.27-0.43 for ENR). (Table 4).

The method specificity was assessed by studying the chromatograms obtained from the sample solution. The method was found to be specific as none of the excipients interfered with the analyte of interest, which is shown in chromatogram Figure 5. Hence the method was found to be suitable for analyzing the commercial drug formulation.

LOD were found to be 0.0505µg/mL for BROM and 0.0611µg/mL for ENR and LOQ were found to be 0.1533µg/mL for BROM and 0.1853µg/mL for ENR (Table 2)

Robustness was studied by taking % variation for ideal system suitability parameter like pH of mobile phase, flow rate and composition of mobile phase. Solution of 100% concentration was prepared and injected in triplicate for every conditions and the % RSD calculated was found to be less than 2 for each conditions (Table 5).

ASSAY OF MARKETED FORMULATION (TABLET)

Total 20 µL sample solution was injected into the chromatographic system and the peak response was measured. The solution was injected three times into the column. The amount present in each tablet was calculated by comparing the areas of test with that of standard and found to be 99.93± 0.38 and 98.17± 9.96 respectively, for BROM and ENR. The results are given in Table 6.

CONCLUSION

A simple, economic, selective and robust RP-HPLC method has been developed and validated for the estimation of Bromhexine HCl and Enrofloxacin in pharmaceutical dosage form. All method validation parameter lie within its acceptance criteria as per ICH Q2 (R1) guideline. So it can be conclude that method is selective, linear, accurate and precise. Hence, it can be successfully used for the routine analysis of Bromhexine HCl and Enrofloxacin in pharmaceutical dosage forms.

ACKNOWLEDGEMENT

The authors are thankful to the management of Nootan Pharmacy College and Sankalchand Patel University Visnagar, Gujarat for providing facilities to carry out research work. Authors are also thankful to Intas Pharmaceuticals Pvt. Ltd. Ahmadabad and Torrent Pharmaceuticals Pvt. Ltd. Ahmadabad for providing the gift samples of Bromhexine HCl and Enrofloxacin.

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