International Journal of Current Trends in Pharmaceutical Research

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Research Article **Open Access**

Development and Validation of RP-HPLC Method for Simultaneous Estimation of Levofloxacin Andambroxol Hydrochloride in Bulk and Pharmaceutical Dosage Form

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A B S T R A C T

A new, precise, rapid, accurate RP-HPLC method was developed for the Simultaneous Estimation of Levofloxacin and Ambroxol HCl in pharmaceutical dosage forms. After optimization the good chromatographic separation was achieved by Isocratic mode with a Mixed phosphate buffer: ACN: methanol (40:40:20v/v%) pH4.5.as the mobile phase with Inertsil ODS C18-250X4.6mm, 5µ,column as stationary phase at flow rate of 1 mL/min and detection wavelength of 223nm.The retention times for Levofloxacin and Ambroxol HCl found to be 2.737min and 4.793min respectively. The linearity of this method was found in the concentration range of 60 µg/mL to 140 µg/mL for Levofloxacin and 9-21 (µg/mL)for Ambroxol HCl. The correlation coefficient R^2value is found to be0.997 for Levofloxacin and 0.995 for Ambroxol HCl. The LOD and LOQ for Levofloxacin were found to be 2.66 mcg µg/mL and 15.69 µg/mL respectively. The LOD and LOQ for Ambroxol HCl were found to be 8.05 µg/mL and 47.55µg/mL respectively. This method was found to be good percentage recovery for Levofloxacin AndAmbroxol HCl were found to be 101.28 and 99.32 respectively indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analyte in the sample without interference from excipients of tablet dosage form. The method was extensively validated according to ICH guidelines for Linearity, Range, Accuracy, Precision, specificity and Robustness.

Keywords: UV spectrophotometer, Levofloxacin and Ambroxol HCl, High performance liquid chromatography.

A R T I C L E I N F O

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Article History: Received 10 March 2017, **Accepted** 15 May 2017, **Available Online** 15 July 2017

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Manuscript ID: IJCTPR3304

PAPER-QR CODE

Citation: Hareesh Dara, *et al.* Development and Validation of RP-HPLC Method for Simultaneous Estimation of Levofloxacin Andambroxol Hydrochloride in Bulk and Pharmaceutical Dosage Form. *Int. J. Curnt. Tren. Pharm, Res.*, 2017, 5(4): 127-133.

1. Introduction

Chromatography: The term chromatography (in Greek Kromatos means colour; Graphos means written) meaning colour writing. The history of chromatography started in eighteenth century by Runge, studying the nature of inorganic compounds on filter paper with great interest. He separated inorganic salts and observed that the inorganic salts travel to different extent producing attractive pattern. A variety of methods are available for separation of components from the mixture and to analyze them. They are broadly classified into physical and chemical methods. These methods are effective in purification, separation and identification of compounds. However difficulty, arises in case of compounds where individual components have very similar physical and chemical properties *i.e.* mixture of liquids having very close boiling points. Similarly, in biological materials, these methods are not satisfactory. Chromatography method represent the most useful and powerful technique for these problems.

High performance liquid chromatography (HPLC)

HPLC is directly derived from classic column chromatography, in which a liquid mobile phase is pumped under pressure rather than by gravity flow through a column filled with a stationary phase. This has resulted in a sharp reduction in separation time, narrower peak zones, and improved resolution. The mobile phase is placed in a solvent reservoir for pumping into the system. A solvent system is usually degassed by vacuum treatment or sonication before use. Most of the drugs in biological sample can be analyzed by HPLC method because of the advantages, such as speed, greater sensitivity (various detectors can be employed), improved resolution (wide variety of stationary phases), reusable columns (expensive columns but can be used for many samples), ideal for the substances of low volatility, easy sample recovery, handling and maintenance, instrumentation lends itself to automation and quantitation (less time and less labour), precise and reproducible, calculations are done by integrator itself and suitable for preparative liquid chromatography on a much large scale.

2. Materials and Methods

Materials:

Levofloxacin, Ambroxol HCl, Potassium dihydrogen phosphate, Methanol, Aectonitrile

Method Development of Levofloxacin and Ambroxol

The selected method was used for method development of the drug molecules Levofloxacin and Ambroxol as per ICH guidelines. By taking different mobile phases and b y changing different columns trials have been done and validation process is done for the optimized method.

Table 1: Optimized chromatographic conditions

Mobile phase	**Phosphate buffer: ACN: Methanol (40:40:20)**
Ph	4.5
Column	Inertsil ODS,C-18, 250×4.6mm ID, 5µm Particle size
Flow rate	1.2 ml/min
Column temperature	Room temperature (20-25^{o}C)
Sample temperature	Room temperature (20-25^{o}C)
Wavelength	223
Injection volume	20 µl
Run time	5 min
Retention time	About 2.737 min for Levofloxacin and 4.793min for Ambroxol

3. Results and Discussions

Validation of the Method

System suitability studies: The suitability of the system was studied by the values obtained for Theoretical plate, Resolution and tailing factor of the chromatogram of standard drugs and presented in the Table-3.The selectivity of the method was revealed by the repeated injection of mobile phase and no interference was found.

Specificity

Specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities, degradants or excipients. A volume of 20 µl of working blank, sample standard solution was injected and the chromatogram was recorded. No peaks were found at retention time of 2.71 and 4.77 min. Hence, the proposed method was specific for Levofloxacin and Ambroxol HCl.

Linearity

Calibration curves for Levofloxacin and Ambroxol HCl were prepared individually. Aliquots of required sample stock solutions were transferred individually to the 10 ml of volumetric flasks and made up to the mark with mobile phase to get concentration of 60, 80, 100, 120, 140 µg/ ml Levofloxacin and 9, 12, 15, 18, 21 µg/ml for Ambroxol HCl respectively. An aliquot (20 µl) of each solution was injected under the operating chromatographic condition as described above and responses were recorded. Calibration curves were constructed by plotting the peak areas versus the concentration and the regression equations were calculated.

Precision

To check the intra-day and inter-day variation of the method, standard concentration was subjected to the proposed HPLC method of analysis. The precision of the proposed method i.e. the intra and inter-day variations in the peak area of the drug solutions was calculated in terms of percent RSD.A statistical evaluation revealed that the relative standard deviation of drugs at different concentration levels for 6 injections was less than 2.0

Accuracy

Accuracy was performed by following standard addition method. In this standard was added to pre-analyzed sample solution.

Standard Stock Solution

An accurately weighed quantity of Levofloxacin (250mg)

and AmbroxolHCl (37 mg) were transferred into a separate 50 ml volumetric flasks and dissolved and diluted to the mark with Mobile phase to obtain standard solutions having concentration of Levofloxacin (5mg/ml) and Ambroxol HCl (0.3mg/ml).

Preparation of sample stock solution:

20 Tablet contents were weighed and triturate to fine powders. Accurately weighed and transferred 400.26 mg of powder equivalent to 500 mg Levofloxacin and 75 mg Ambroxol HCl into a 100 ml volumetric flask. To that 10 ml mobile phase is added to dissolve and then the volume is making up with mobile phase. Then it is filtered with 0.45 micron filter and it is sonicated. From this stock solution 1ml is pipette out and transferred to 50 ml volumetric flask and volume is makeup to 50 ml to prepare 100µg/ml and 30µg/ml final mixed concentrations of Levofloxacin and Ambroxol HCl respectively

Procedure: Sample solutions prepared separately by addition of standard stock at 80 %, 100 % and 120 % of working sample concentration was injected three times into the chromatographic system.

Robustness

Robustness examines the effect of variation in operational parameters on the analysis results. For the determination of a method's robustness, chromatographic parameters like detector wavelength and flow rate are varied within a realistic range and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range.

Ruggedness

Three assay samples of drug product at 100% of the working sample concentration were prepared and injected into the chromatographic system by different analysts.

Acceptance Criteria: % RSD of areas of 2 sample injections in each set should be not more than 2.0.

Overall % RSD of areas of 4 sample injections (set-1 and set-2) should be not more than 2.0.

Limit of Detection And Limit of Quantification: LOD and LOQ were determined by using the formula based on the standard deviation of the response and the slope.LOD and LOQ were calculated by using equations, LOD=3.3 X /S and LOQ =10 X /S,

Acceptance Criteria: % RSD of areas of 2 sample injections in each set should be not more than 2.0.

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Where,

is the standard deviation,

S is the slope of the calibration curve.

Calculation of LOD & LOQ

Formula for LOD: **3.3Xσ/S**

Formula for LOQ: **10 Xσ/S**

Where σ = Standard deviation, S = Slope

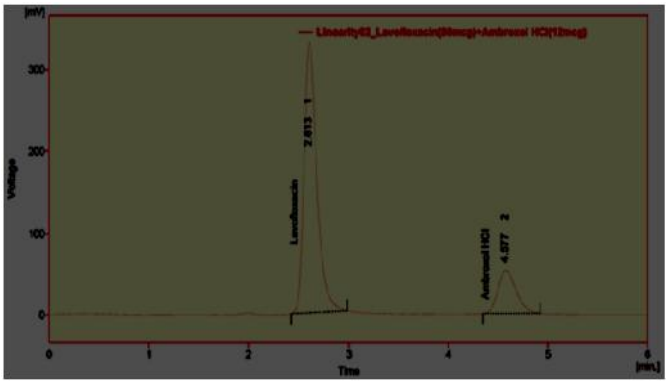

Figure1: Chromatogram of system suitability data

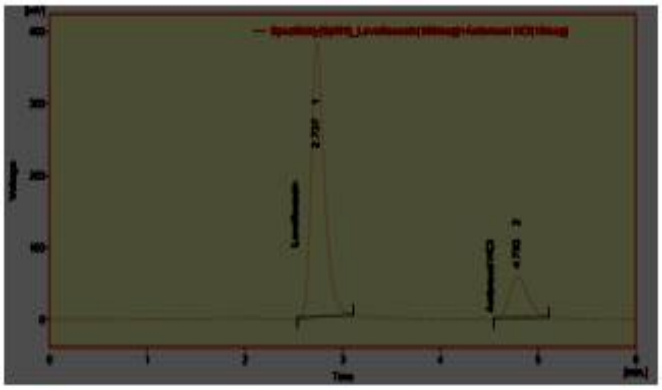

Figure 2: Chromatogram of specificity sample

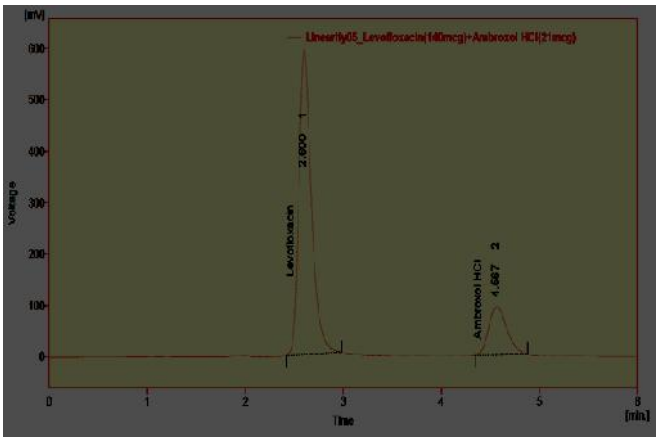

Figure 3: Linearity Chromatogram

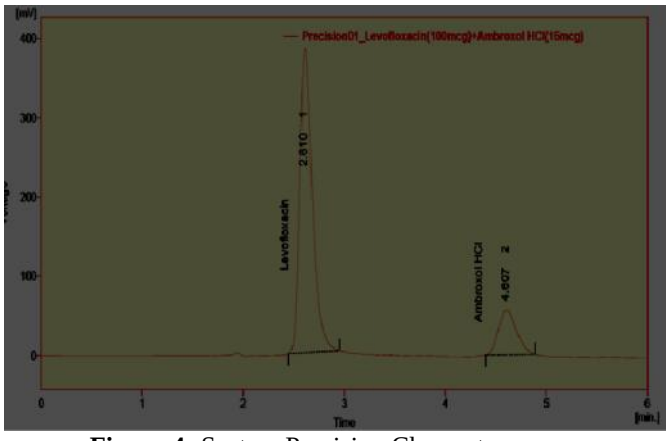

Figure 4: System Precision Chromatogram

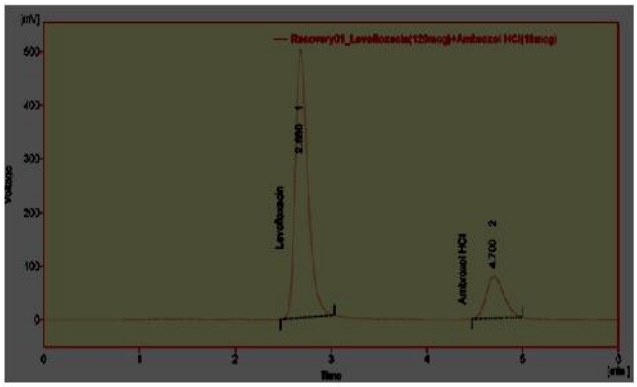

Figure 5: Chromatogram for Accuracy 100%

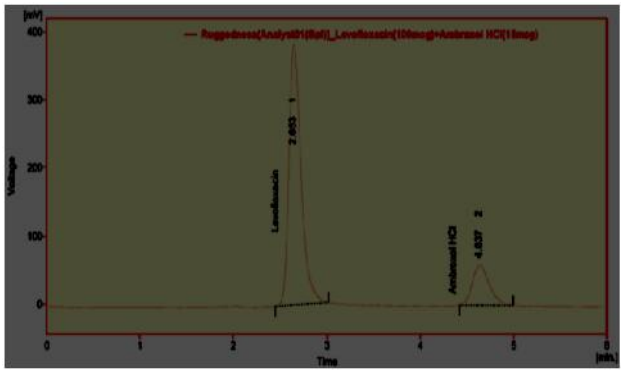

Figure 7: Chromatograms of ruggedness sample (analyst-1)

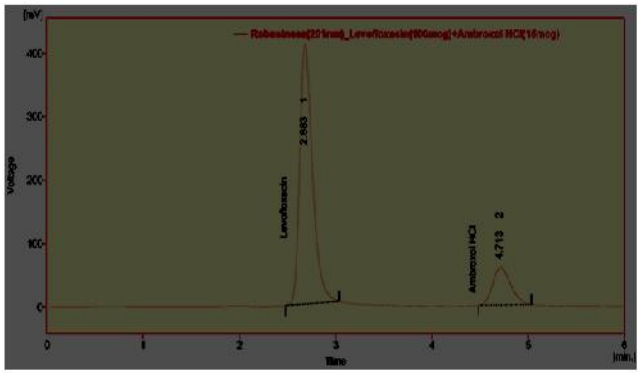

Figure 6: Chromatogram of Low Wavelength: (201nm)

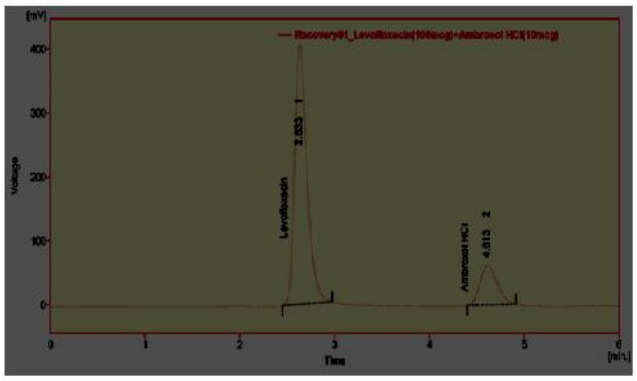

Figure 8: Chromatogram of LOD and LOQ

Table 2: System suitability data of Levofloxacin and Ambroxol HCl

parameter	Levofloxacin	Ambroxol HCl	Acceptance criteria
Retention time	2.6	4.57	
Theoretical plates	2146	2930	>2500
Tailing factor	1.62	1.40	<2.00
% RSD	0.12	0.10	<2.00

Table 3: Specificity Data of Sample

S. No	Name	Retention time (min)	Area (mV. s)	Efficiency (th.pl)	Asymmetry	Resolution
1	Levofloxacin	2.73	3544.8	2020	1.62	6.850
2	Ambroxol HCl	4.79	751.10	2886	1.48	

Table 4: Specificity Data of Standard

S. No	Name	Retention time (min)	Area (mV .s)	Efficiency (th.pl)	Asymmetry	Resolution
1	Levofloxacin	2.71	3541.48	2990	1.62	6.915
2	AmbroxolHCl	4.77	735.45	2955	1.49	

Table 5: Data of Linearity

S.No	Levofloxacin		Ambroxol HCl	
	Working conc. (µg/ml)	Peak area	Working conc. (µg/ml)	Peak area
1	60	2281.504	9	532.639
2	80	2945.983	12	677.273
3	100	3751.285	15	823.701
4	120	4529.888	18	988.733
5	140	5417.729	21	1187.091
Correlation Coefficient (R^2)		0.9977	0.9953	

Slope	39.282	54.012
Intercept	-142.9	31.705

Table 6: Precision Data for Levofloxacin and Ambroxol HCl

Injection No	**Levofloxacin**		**AmbroxolHCl**	
	Retention time (min)	**Peak area**	**Retention time (min)**	**Peak area**
1	2.61	3566.38	4.60	748.38
2	2.65	3560.50	4.63	732.78
3	2.63	3551.05	4.61	740.22
4	2.63	3541.02	4.60	727.18
5	2.62	3544.77	4.58	740.06
6	2.63	3504.75	4.61	719.41
Mean	2.62	3544.75	4.60	734.67
SD	0.0145	21.769	0.016	10.394
% RSD	0.55	0.61	0.35	1.41

Table 7: Summary of Accuracy data

Sample	**Accuracy**	**Amount Added** (µg/ml)	**Standard Amount** (µg/ml)	**Amount Recovered mg**	**Mean % Recovery**
Levofloxacin	80 %	80	20		MEAN= 100.99 %
	80 %	80	20	100.99	
	80 %	80	20		
	100 %	100	20		MEAN= 101.45 %
	100 %	100	20	121.74	
	100 %	100	20		
	120 %	120	20		MEAN= 101.39 %
	120 %	120	20	141.95	
	120 %	120	20		
Ambroxol HCL	80%	12	3		MEAN= 98.35 %
	80 %	12	3	14.75	
	80 %	12	3		
	100 %	15	3		MEAN= 100.63 %
	100 %	15	3	18.11	
	100 %	15	3		
	120 %	18	3		MEAN= 99.03 %
	120 %	18	3	20.80	
	120 %	18	3		

Table 8: Robustness Data for Flow Rate Variation

S.No	Flow rate (ml/min)	Levofloxacin			Ambroxol Hcl		
		RT (min)	Efficiency (th.pl)	Asymmetry	RT (min)	Efficiency (th.pl)	Asymmetry
1	0.8	3.13	2125	1.68	5.48	3056	1.49
2	1.2	2.3	1945	1.58	4.1	2751	1.38

Table 9: Robustness Data for Effect of Wavelength Variation

S.No	Wave length (nm)	Desvenlafaxine succinate			Clonazepam		
		RT (min)	Efficiency (th.pl)	Asymmetry	RT (min)	Efficiency (th.pl)	Asymmetry
1	Low 201	2.68	2942	1.62	4.71	2882	1.46
3	High 205	2.7	2071	1.67	4.7	2011	1.42

Table 10: Ruggedness Data for Levofloxacin and Ambroxol HCl

S.No	Sample area			
	Analyst -1 (set-1)		Analyst -2 (set-2)	
	Levofloxacin	Ambroxol HCl	Levofloxacin	Ambroxol HCl
1	3435	756	3417	710
2	3351	728	3435	730
Mean	3392	747	3426	720
SD	53.39	19.79	12.72	14.14
%RSD	1.5	2.0	0.37	1.9

Table 11: Data Table of LOD & LOQ for Levofloxacin and Ambroxol HCl

S.No	DRUG	LOD (µg/ml)	LOQ (µg/ml)
1	Levofloxacin	2.66µg/ml	0.29µg/ml
2	AmbroxolHCl	8.05µg/ml	0.88µg/ml

4. Conclusion

From the reported literature, there were few methods established for the determination of Levofloxacin and Ambroxol HCL in individual and in combination with other drug. The scope and objective of the present work is to develop and validate a new simple RP-HPLC method for simultaneous estimation of Levofloxacin and Ambroxol HCL in bulk and pharamceutical dosage form. In simultaneous RP-HPLC method development, shimadzu prominence HPLC with UV detector and column used is C_{18} Inertsil ODS (250 X 4.6mm) column with 5-micron particle size. Injection volume of 20 µL is injected and eluted with the mobile phase selected after optimization was mixed Phosphate buffer, Acetonitrile and Methanol in the ratio of 40:40:20 was found to be ideal. The flow rate was found to be optimized at 1.0 mL/min. Detection was carried out at 223 nm. Quantitation was done by external standard method with the above mentioned optimized chromatographic condition. This system produced symmetric peak shape, good resolution and reasonable retention times of Levofloxacin and Ambroxol HCL were found to be 2.737 and 4.793 minutes respectively. The showed linearity Levofloxacin and Ambroxol HCL in the range of 60-140 µg/mL and 9-21 µg/mL respectively. The slope, intercept and correlation coefficient values for Levofloxacin were found to be 39.28, -142.9, and 0.997 respectively and 54.01, +31.70and 0.995 respectively for Ambroxol HCL which indicates excellent correlation between response factor Vsconcentration of standard solutions. Precision of the developed method was studied under system precision and method precision. The %RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust. The % RSD value for percentage recovery Levofloxacin and Ambroxol HCL was found to be within the acceptance criteria. The results indicate satisfactory accuracy of method for simultaneous estimation of the Levofloxacin and Ambroxol HCL. Good agreement was seen in the assay results of pharmaceutical formulation by developed method. Hence it can be concluded that the proposed method was a good approach for obtaining reliable results and found to be suitable for the routine analysis of Levofloxacin and Ambroxol HCL in bulk and pharmaceutical formulation.

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