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STABILITY INDICATING RP-HPLC METHOD FOR COMBINATION OF AMBROXOL HYDROCHLORIDE AND LEVOFLOXACIN HEMIHYDRATE IN PHARMACEUTICAL FORMULATION

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Keywords:

Ambroxol hydrochloride (AMB), Levofloxacin hemihydrate (LVF), Stability indicating method, Assay, Reverse Phase - High Performance Liquid Chromatography (RP - HPLC)

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ABSTRACT: Ambroxol hydrochloride (AMB) and Levofloxacin hemihydrate (LVF) in combination was separated using Reverse Phase - High Performance Liquid Chromatographic (RP-HPLC) method. Mobile phase acetonitrile and 0.05 M potassium di-hydrogen ortho phosphate buffer (pH 7.0 adjusted with sodium hydroxide solution) (50: 50, v/v) was selected for this chromatographic method. The separation was achieved in Zorbax Eclipse XDB -C18 column with (250 × 4.5 mm i.d), 5µm particle size with flow rate of 1.0 ml / min. At 248 nm wavelength 10 µl of 60 µg/ml Ambroxol hydrochloride and 400 µg/ml Levofloxacin hemihydrate (LVF) was injected for 15 min run time and individual peak was obtained for LVF at retention time 2.61 min and for AMB at retention time 7.69 min. Linearity was achieved for Ambroxol hydrochloride in the range of 48 mcg/ml to 72mcg/ml and for Levofloxacin hemihydrate in the range of 320 mcg/ml to 480 mcg/ml. For stress degradation AMB and LVF were subjected to acid hydrolysis, base hydrolysis, thermal degradation, UV light degradation, oxidation and analysed with this chromatographic method. The results obtained with this method are useful for assay of this pharmaceutical formulation; hence, this method can be used in pharmaceutical industry.

INTRODUCTION: Ambroxol hydrochloride and Levofloxacin hemihydrate in combination used for the treatment and relief of symptoms of both upper and lower respiratory tract infections. Ambroxol hydrochloride is a mucolytic agent. Its IUPAC name is Trans- 4- [(2-amino-3, 5-dibromobenzyl) amino] cyclohexanol HCl **Fig. 1**. [1, 2] Levofloxacin hemihydrate is a fluoroquinolone antibacterial agent. Its IUPAC name is t(-)-(S)-9 fluoro-2, 3-dihydro- 3- Methyl- 10- (4-methyl piperazin-1yl) -7-oxo -7H-pyrido [1, 2, 3 de]-1, 4-benzoxazine-6-carboxylic acid hemihydrates **Fig. 2**. [3, 4]

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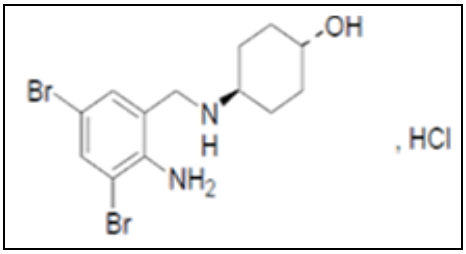

FIG. 1: AMBROXOL HYDROCHLORIDE

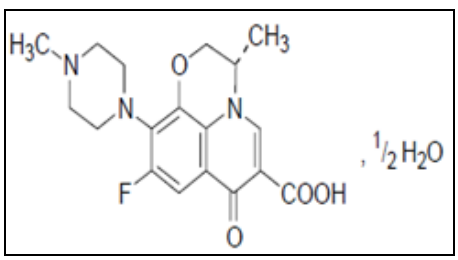

FIG. 2: LEVOFLOXACIN HEMIHYDRATE

This marketed formulation is available in 75 mg of Ambroxol hydrochloride and 500 mg of Levofloxacin hemihydrate dose.

To develop such an accurate, sensitive, rapid, precise and economical method for routine analysis is inevitable of Ambroxol hydrochloride and Levofloxacin hemihydrate combination in their pharmaceutical dosage form successfully.

MATERIALS AND METHODS:

Instrumentation: Agilent technologies 1260 infinity module HPLC system with photo diode array detector was used. Analytical balance Mettler Toledo model MS105DU was used to weigh chemicals. pH meter Systronics- model µpH system 361 was used to measure pH.

Reagents and Chemicals: Acetonitrile and methanol of HPLC grade, potassium dihydrogen phosphate of analytical reagent grade were procured from Merck Pvt. Ltd., India. Rankem Pharmaceuticals India Ltd., was supplied analytical grade sodium hydroxide, hydroxide peroxide, hydrochloride acid.

Preparation of Buffer: Accurately measured potassium di-hydrogen ortho phosphate buffer 6.8 gm was dissolved in 1000 ml of HPLC grade water pH was adjusted to 7 with the help of sodium hydroxide solution.

Mobile Phase Preparation: Mobile phase was prepared by taking equal amount (50:50) of prepared buffer solution and acetonitrile. It was sonicated for 20 min to degas in the mixture mobile phase. The obtain solution was further used in the analysis.

Standard Preparation: Weighed accurately 75 mg of Ambroxol hydrochloride and 500 mg of Levofloxacin hemihydrate and transferred in to 50 ml volumetric flask. Added 25 ml of mobile phase. Sonicated to dissolve. Made volume upto mark with mobile phase and mixed. (1500 µg/ml AMB and 10000 µg/ml LVF). Pipetted 10 ml of above solution into 100 ml volumetric flask, made volume upto the mark with mobile phase and mixed well. (150 µg/ml AMB and 1000 µg/ml LVF). Further pipetted 10 ml of above solution into 25 ml volumetric flask, made volume upto the mark with mobile phase and mixed well. (60 µg/ml AMB and 400 µg/ml LVF).

Sample Preparation: Weighed twenty tablets and crushed them into fine powder with the help of mortar and pestle. Weighed and transferred sample powder equivalent to 75 mg AMB and 500 mg LVF into 50 ml volumetric flask. Added 10 ml mobile phase and sonicated for 10 min with continuous shaking. Made volume upto mark with mobile phase and mixed well and filtered through Whatmann filter paper (0.45 µ). (1500 µg/ml AMB and 10000 µg/ml LVF). Further pipetted 10 ml of above filtrate into 100 ml volumetric flask, made volume upto the mark with mobile phase and mixed well. (150 µg/ml AMB and 1000 µg/ml LVF). Further pipetted 10 ml of above solution into 25 ml volumetric flask, made volume upto the mark with mobile phase and mixed well. (60 µg/ml AMB and 400 µg/ml LVF).

Wavelength Selection: Solution of 60 µg/ml AMB and 400 µg/ml LVF were scanned in the UV region of 200-400 nm at room temperature and the obtain spectra was by software **Fig. 3**.

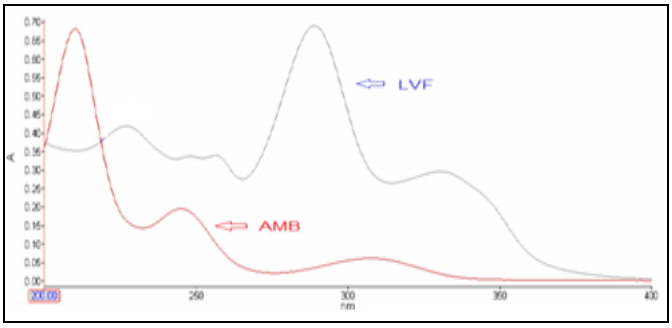

FIG. 3: OVERLAY ZERO ORDER UV SPECTRUM OF AMB AND LVF IN DILUENTS

Chromatography Method Development: Various trials were made with reference of methods used for assay of anti-histamine drugs. In each trial peak shape, resolution between two components and tailing factor were observed. Buffer and acetonitrile in different proportions were tried and finally 0.05 M potassium dihydrogen ortho phosphate with pH 7 adjusted with sodium hydroxide solution and acetonitrile in the ratio of 50: 50% v/v selected with better peak shape and resolution. In mobile phase both the drugs were found to be soluble and stable so mobile phase is selected as a diluent. Chromatographic method selected with a Eclipse XDB column - C18, (250 × 4.6 mm i.d) and particle size 5 µm. AMB and LEV peak is obtained at 7.69 min and 2.61 min respectively with flow rate of 1.0 ml/min and injection volume 10 µl for 15 min run time. The method was further validated under the chromatographic conditions.

Method Validation: The established chromatographic method was validated in compliance with ICH guidelines. The parameters like system suitability along with precision, linearity, specificity and accuracy, LOQ and LOD were performed for validation.

Forced Degradation Studies (FDS):

Degradation with 3% H_2O_2: Accurately weighed 1118 mg of sample powder and transferred in to 50 ml volumetric flask. Added 10 ml mobile phase and sonicated for 10 min with continuous shaking. Added 5.0 ml 3% v/v hydrogen peroxide to the flask. Kept the volumetric flask at 60 °C for 12 h. After the specified time remove the flask from water bath and allow to cool the flask at room temperature. Made volume upto the mark with mobile phase and mixed well. Filtered the solution with 0.45 µm PVDF filter. Further pipetted 10 ml of above filtrate into 100 ml volumetric flask, made volume upto the mark with mobile phase and mixed well. Further dilution was made by taking 10 ml previous solution to 25 ml with mobile phase. (400 µg/ml LVF and 60 µg/ml AMB).

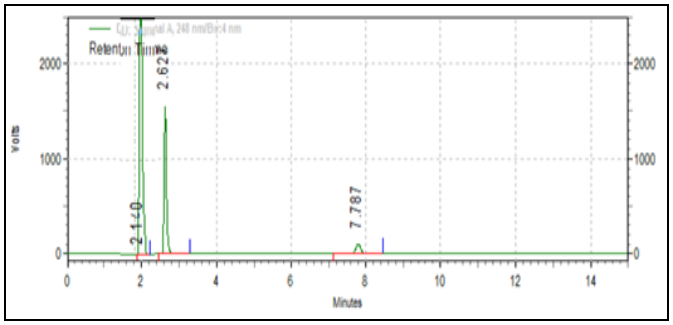

FIG. 4: CHROMATOGRAPH OF FDS ON SAMPLE SOLUTION CONTAINING AMBROXOL HYDROCHLORIDE AND LEVOFLOXACIN HEMIHYDRATE USING 3% HYDROGEN PEROXIDE SOLUTION

Degradation with 0.1M HCl: Accurately weighed 1118 mg of sample powder and transferred in to 50 ml volumetric flask added 10 ml mobile phase and sonicated for 10 min with continuous shaking. Added 5.0 ml 0.1 M hydrochloric acid to the volumetric flask. Store flask at 60 °C for 12 h. After the specified time remove the flask from water bath and allow to cool the flask at room temperature. Added 5 ml 0.1 M sodium hydroxide. Made volume upto the mark with mobile phase and mixed well. Filtered the solution with 0.45 µm PVDF filter. Further pipetted 10 ml of above filtrate into 100 ml volumetric flask, made volume upto the mark with mobile phase and mixed well. Further dilution was made by taking 10 ml previous solution to 25 ml with mobile phase. (400 µg/ml LVF and 60 µg/ml AMB).

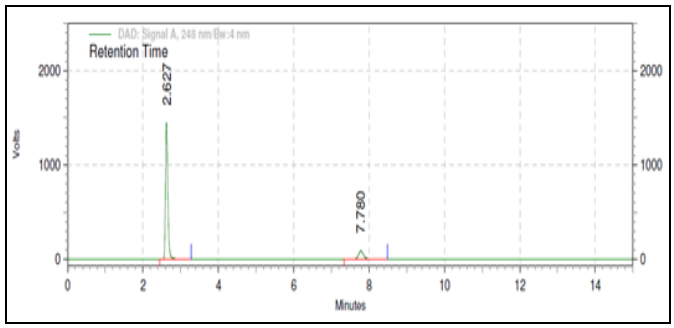

FIG. 5: CHROMATOGRAPH OF FDS ON SAMPLE SOLUTION CONTAINING AMBROXOL HYDROCHLORIDE AND LEVOFLOXACIN HEMIHYDRATE USING 0.1 M HCl SOLUTION

Degradation with 0.1M NaOH: Accurately weighed 1118 mg of sample powder and transferred in to 50 ml volumetric flask added 10 ml mobile phase and sonicated for 10 min with continuous shaking. Added 5 ml 0.1 M sodium hydroxide to the volumetric flask. Store flask at 60°C for 12 h. After the specified time remove the flask from water bath and allow to cool the flask at room temperature. Added 5 ml 0.1 M hydrochloric acid. Made volume upto the mark with mobile phase and mixed well. Filtered the solution with 0.45 µm PVDF filter. Further pipetted 10 ml of above filtrate into 100 ml volumetric flask, made volume upto the mark with mobile phase and mixed well. Further dilution was made by taking 10 ml previous solution to 25 ml with mobile phase. (400 µg/ml LVF and 60 µg/ml AMB).

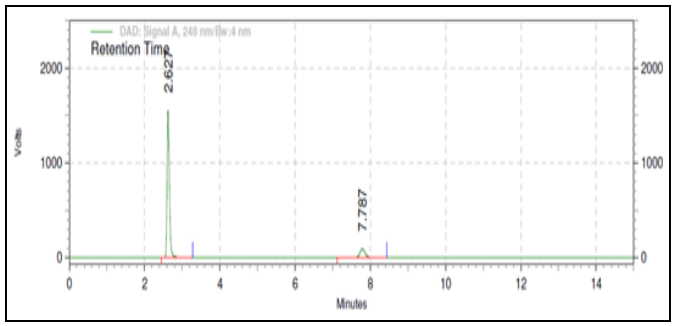

FIG. 6: CHROMATOGRAPH OF FDS ON SAMPLE SOLUTION CONTAINING AMBROXOL HYDROCHLORIDE AND LEVOFLOXACIN HEMIHYDRATE USING 0.1 M NaOH SOLUTION

Exposed to Heat: Accurately weighed 1118 mg of sample powder and transferred in to 50 ml

volumetric flask Added 10 ml Mobile phase and sonicated for 10 min with continuous shaking. Volumetric flask exposed under heat at 80 ºC for 12 h. After the specified time remove the flask from heat and allow to cool the flask at room temperature. Made volume upto the mark with mobile phase and mixed well. Filtered the solution with 0.45 µm PVDF filter. Further pipetted 10 ml of above filtrate into 100 ml volumetric flask, made volume upto the mark with mobile phase and mixed well. Further dilution was made by taking 10 ml previous solution to 25 ml with mobile phase. (400 µg/ml LVF and 60 µg/ml AMB).

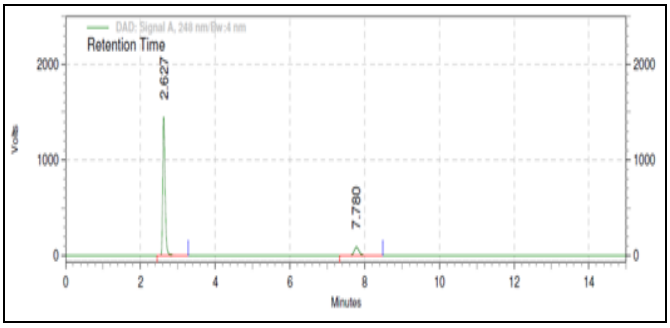

FIG. 7: CHROMATOGRAPH OF FDS ON SAMPLE SOLUTION CONTAINING AMBROXOL HYDRO-CHLORIDE AND LEVOFLOXACIN HEMIHYDRATE USING HEAT

Exposed to UV Light: Accurately weighed 1118 mg of sample powder and transferred in to 50 ml volumetric flask added 10 ml mobile phase and sonicated for 10 min with continuous shaking. Volumetric flask exposed for UV radiation for 12h. After the specified time made volume upto the mark with mobile phase and mixed well. Filtered the solution with 0.45 µm PVDF filter. Further pipetted 10 ml of above filtrate into 100 ml volumetric flask, made volume upto the mark with mobile phase and mixed well.

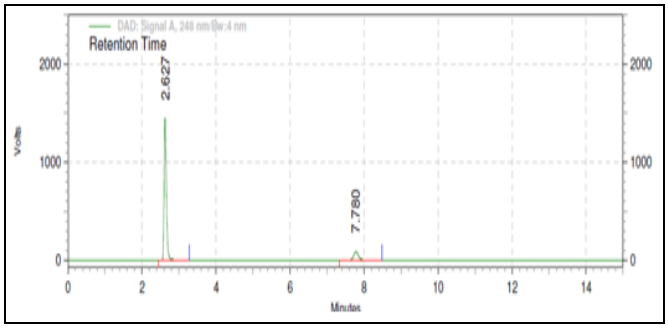

FIG. 8 CHROMATOGRAPH OF FDS ON SAMPLE SOLUTION CONTAINING AMBROXOL HYDRO-CHLORIDE AND LEVOFLOXACIN HEMIHYDRATE USING UV LIGHT

Further dilution was made by taking 10 ml previous solution to 25 ml with mobile phase. (400 µg/ml LVF and 60 µg/ml AMB).

RESULTS AND DISCUSSION:

System Suitability: The system suitability was performed on standard solution (60 µg/ml AMB and 400 µg/ml LVF).

TABLE 1: SYSTEM SUITABILITY TEST PARAMETER

System suitability parameters	Proposed Method	
	AMB	LVF
Retention times (R_t) (min)	7.69 ± 0.004	2.61 ± 0.002
Theoretical plates (N)	15818	7893
Resolution (R_S)	28.12 ± 0.02	
Tailing factor (A_S)	1.16 ± 0.01	1.33 ± 0.03
Capacity Factor	75.91 ± 0.04	25.12 ± 0.02

All system suitability parameters were obtained by six repeated injections and all the parameters were found to be within specified limits.

Specificity: Specificity was performed by determining peak purity. There were no peaks was observed to interfere with our analytes.

Linearity: Linearity was developed by generating graph between analyte sample concentration versus average peak area of the analytes. Data as per **Table 2** and graphically represented in Graph **Fig. 9** and **10** indicate that the linearity for AMB was 48 to 72 (µg/ml) and for LVF was 320 to 480 (µg/ml).

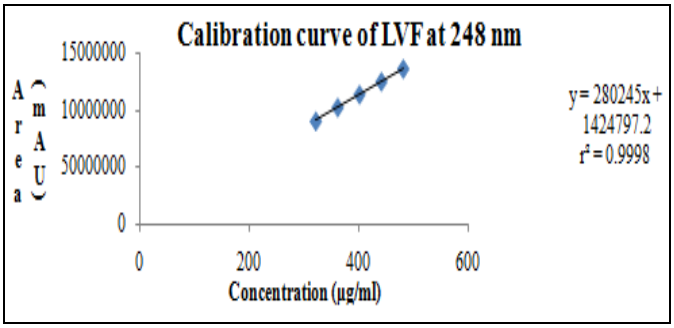

FIG. 9: CALIBRATION CURVE OF LVF AT 248 nm

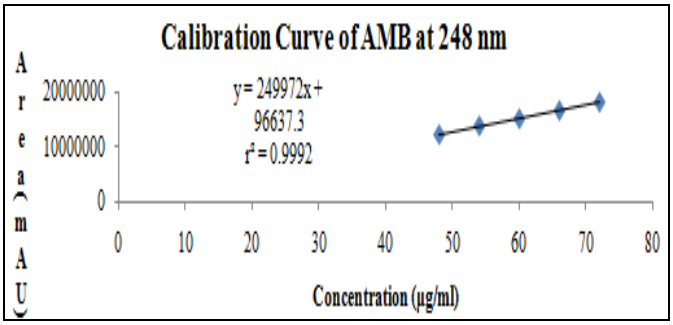

FIG. 10: CALIBRATION CURVE OF AMB AT 248 nm

TABLE 2: LINEARITY

Linearity level (%)	Final conc. (µg/ml)		Mean area	
	LVF	AMB	LVF	AMB
80	320	48	90870100	12067069
90	360	54	102705557	13714018.5
100	400	60	113847199	15045227.5
110	440	66	124847199	16585421.5
120	480	72	135904318	18138025

Accuracy: Accuracy (recovery) study was performed on known amount of placebo by spiking in API. The Samples were prepared by adding 80% to 120% of the sample concentration. As per the data it was indicate that the method has an acceptable level of accuracy.

Precision:

System Precision: The standard solution of AMB (60 ppm) and LVF (400 ppm) were injected into the HPLC system with the same condition for six times. The % Relative standard deviation for six samples were found to be in the limits.

Method Precision: By analysing assay for six individual samples prepared from same batch precision test was evaluated for the proposed method. The average % assay and the % RSD (Relative Standard Deviation) for the six sample preparation were found to be in the specified limits.

Intermediate Precision (Ruggedness): Intermediate precision of method was performed on various HPLC, columns and analyst on different days. Six samples of thee standard solution of AMB (60 ppm) and LVF (400 ppm of same batch were prepared and analysed. The mean, SD, and % relative standard deviation for the two sets of data are shown in **Table 5**.

TABLE 3: ACCURACY

Accuracy	Theoretical amount (ppm)		Practical amount (ppm)		% Recovery		Mean	
	AMB	LVF	AMB	LVF	AMB	LVF	AMB	LVF
80 %	48.0	320.0	47.9	319.3	99.8	99.8	100.1	100.0
	48.0	320.0	48.0	320.0	100.1	100.0		
	48.0	320.0	48.2	320.4	100.4	100.1		
100%	60.0	400.0	60.0	400.4	100.0	100.1	99.7	100.3
	60.0	400.0	59.7	401.0	99.5	100.2		
	60.0	400.0	59.7	401.6	99.5	100.4		
120 %	72.0	480.0	71.8	480.2	99.7	100.1	99.6	100.4
	72.0	480.0	71.7	477.0	99.6	99.4		
	72.0	480.0	71.7	489.2	99.6	101.9		

TABLE 4: METHOD PRECISION

Concentration AMB (60 ppm) LVF (400 ppm)	Samples	Area of Peak		% Assay	
		AMB	LVF	AMB	LVF
	1	15013152	114250211.5	99.5	100.8
	2	15065452	112288127.5	100	99.2
	3	15007408.5	111844332.5	99.8	99.0
	4	15034188.5	112089892.0	99.7	99.0
	5	14993626	111729915.0	99.6	98.8
	6	15132744.5	112906558.0	100.4	99.8
	Mean			99.8	99.4
	% RSD			0.33	0.76

TABLE 5: INTERMEDIATE PRECISION

Concentration AMB (60 ppm) LVF (400 ppm)	Samples	Area		%Assay	
		AMB	LVF	AMB	LVF
	1	15013152.0	113636792	99.5	100.2
	2	15065452.0	112011324	99.7	98.7
	3	15007408.5	112027548	99.3	98.7
	4	15034188.5	111906677	99.6	98.7
	5	14993626.0	112006719	99.3	98.8
	6	15132744.5	113519978	100.2	100.1
	Mean			99.6	99.2
	% RSD			0.34	0.74
% Difference between Method Precision and Intermediate Precision				0.2	0.2

Robustness: Robustness of the method was investigated **Table 6** by varying the analytical estimation operating conditions such as flow rate of mobile phase (± 0.2 ml / min), column oven temperature (± 2% ºC), and pH of buffer (0.2). The standard solution of AMB (60 ppm) and LVF (400 ppm) was prepared as per the standard method described above and estimated as per the developed procedure.

Stability of Sample Solution: The Prepared sample solution was stable up to 24 h. The Data for Forced degradation are tabulated in **Table 7**. From blank and placebo, the interference was negligible in the analyte peaks, Peak purity of all FD samples were obtained and found in specified limit. The obtained data reveals that the developed method was highly specific and stability indicating for the simultaneous estimation of Ambroxol hydrochloride and Levofloxacin hemihydrate, in their marketed tablet formulation.

Forced Degradation Study: For this chromatographic method following data was obtained for forced degradation study.

TABLE 6: ROBUSTNESS STUDY FOR AMB AND LVF

Factors		Retention time (min)		Asymmetry (A_s)		Resolution
		AMB	LVF	AMB	LVF	
pH of mobile phase	6.8	7.688	2.610	1.132	1.290	28.49
	7.0	7.690	2.610	1.160	1.330	28.12
	7.2	7.691	2.611	1.141	1.286	28.50
	Mean ± SD	7.689 ± 0.001	2.610 ± 0.000	1.144 ± 0.014	1.302 ± 0.024	28.37 ± 0.216
Temp (ºC)	28	7.449	2.591	1.139	1.297	27.53
	30	7.690	2.610	1.160	1.330	28.12
	32	7.890	2.627	1.142	1.283	29.29
	Mean ± SD	7.676 ± 0.220	2.609 ± 0.018	1.147 ± 0.011	1.303 ± 0.024	28.31± 0.895
Flow rate	0.8	9.584	3.313	1.157	1.337	30.24
	1	7.690	2.610	1.160	1.330	28.12
	1.2	6.403	2.175	1.136	1.249	26.82
	Mean ± SD	7.890 ± 1.600	2.699 ± 0.574	1.151 ± 0.013	1.305 ± 0.048	28.39 ± 1.726

TABLE 7: SOLVENT STABILITY

Time points (h)	Ambroxol hydrochloride % Difference		Levofloxacin hemihydrate % Difference	
	Standard solution	Test solution	Standard solution	Test solution
Initial	Nil	Nil	Nil	Nil
6	0.25	0.27	0.59	0.29
12	0.45	0.42	0.02	0.61
18	0.43	0.42	0.75	0.76
24	0.54	0.45	0.37	1.01

TABLE 8: SUMMARY OF VALIDATION PARAMETERS OF RP-HPLC

Parameters	AMB	LVF
Recovery %	99.6 – 100.1	100.0 – 100.4
Method precision	0.33	0.76
Intermediate precision	0.34	0.74
Specificity	No interference	No interference
Solvent suitability	24 hr	24 h

TABLE 9: ANALYSIS OF MARKETED TABLET FORMULATION

Brand name	API	Label claim (mg)	Amount taken (µg/ml) (n = 3)	Amount found (µg/ml) (n = 3)	% Label claim ± S.D
Lebact - AM	AMB	75	60	59.95	99.92 ± 0.05
	LVF	500	400	396.64	99.16 ± 1.05

TABLE 10: FD STUDY

S. no.	Condition	Area		% Assay		% Degradation	
		Ambroxol hydrochloride	Levofloxacin hemihydrate	Ambroxol hydrochloride	Levofloxacin hemihydrate	Ambroxol hydrochloride	Levofloxacin Hemihydrate
1	As such Sample	14979073	111889522	99.20	98.66	---	---
2	0.1 M HCl 80 ºC 1 h	12908357	109253623	85.49	96.34	14.51	3.66
3	0.1 M NaOH RT 1 h	13907253	99072572	92.10	87.36	7.90	12.64
4	3 % H_2O_2 RT 1 h	12083463	92537538	80.03	81.60	19.97	18.40
5	Heat Treatment	13298352	98024742	88.07	86.44	11.93	13.56
6	UV light treatment	13830273	96375374	91.60	84.98	8.40	15.02

CONCLUSION: It is concluded that the developed stability indicting analytical RP-High Performance Liquid Chromatography method is highly fast, very sensitive, enough economical and reliable and is comply with all validation parameter ad per ICH guidelines. So, for routine estimation of tablets containing AMB and LVF this method can be employed.

Due to reduction of cost of analysis and time and more effective than reported analytical methods may be replaced by the our developed stability indicating Reverse phase high performance liquid chromatography method.
This validated method can be used for faster samples testing routinely in QC lab.

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CONFLICT OF INTEREST: Nil

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