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Method Development and Validation for Quantitative Estimation of Levofloxacin Hemihydrate and Bromhexine HCl in Oral Solution Dosage Form by RP-HPLC Method

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Keywords: RP-HPLC, Levofloxacin hemihydrate, and Bromhexine HCL, Method Development, Validation, ICH guidelines.

ABSTRACT

A new and simple high-performance liquid chromatography (HPLC) method based on separation has been developed for the quantitative determination of levofloxacin and Bromhexine in combination. The chromatographic separation was achieved on the Thermo Scientific Synchronise C18 column (150mm x 4.6mm x i.d. 5µ). The mobile phase selected was 25mM potassium dihydrogen orthophosphate buffer adjusted to pH-3.0 with orthophosphoric acid and methanol in the ratio of 35:65 v/v at flow rate 0.8 ml/min with column temperature maintained at 45°C and 10µl injection volume. The detection was carried out at 254nm. The diluent used was Methanol and the run time kept for the method was 10 minutes. The retention time of levofloxacin hemihydrate and bromhexine HCl was found to be 2.38 minutes and 3.00 minutes respectively. The method was linear over the range of 50-400µg/ml for levofloxacin hemihydrate and 3.75-30µg/ml for bromhexine HCl. The regression coefficient obtained for levofloxacin hemihydrate and bromhexine HCl was 0.9995and0.9995respectively. The method was validated as per ICH guidelines and the study reveals that the developed method is specific, rapid, reliable, and reproducible hence it can be applied for routine quality control analysis of bromhexine HCl and levofloxacin hemihydrate in combination.

INTRODUCTION

Levofloxacin is a third-generation antibiotic. It is twice as active as its isomer ofloxacin, effective against a number of Gram-positive, Gram-negative, and specifically effective against the organisms that cause atypical pneumonia. Levofloxacin is one of the so-called respiratory.

Bromhexine is a mucolytic agent used in the treatment of respiratory disorders associated with viscid or excessive mucus. Acute respiratory ailments, fowl cholera, enteritis, coryza, dermatitis, etc. can be successfully treated using Veterinary Levofloxacin & Bromhexine Solution Oral Solution. The chemical structure of the drugs was represented in Figures no.1 & 2 respectively. [1]

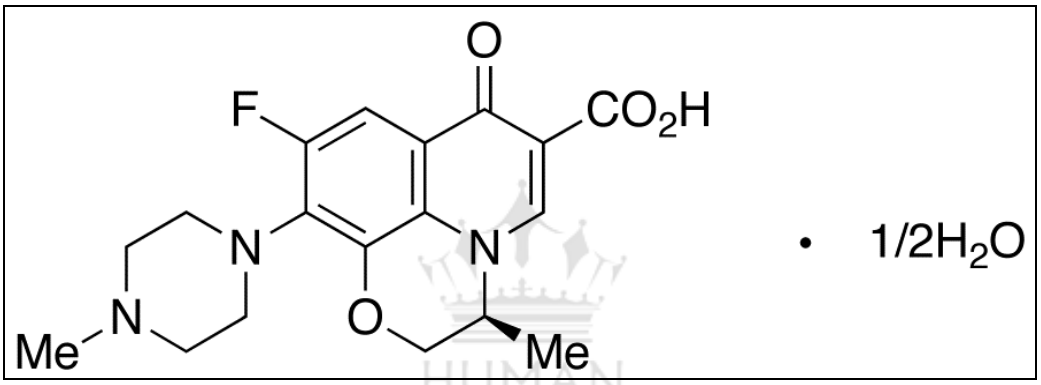

Figure No. 1- Structure of Levofloxacin hemihydrate

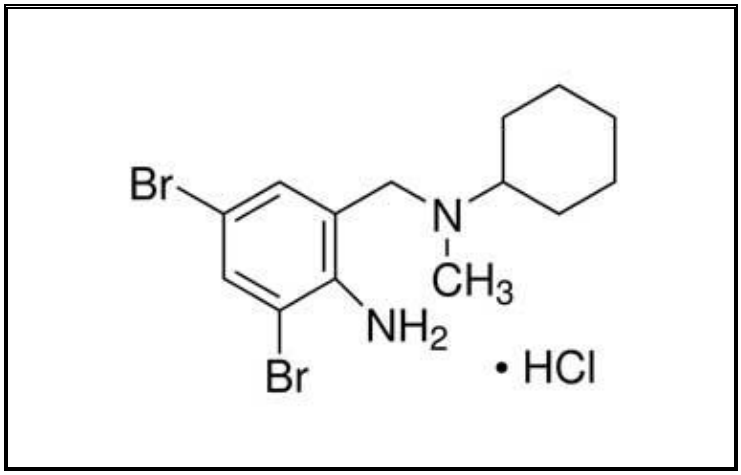

Figure No. 2- Structure of Bromhexine HCl

The chemical name of Levofloxacin hemihydrate is (2*S*)-7-fluoro-2-methyl-6-(4-methyl piperazine-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.0^{5,13}]trideca-5(13),6,8,11-tetraene-11-carboxylic acid; hydrate.[(2)]Levofloxacin hemihydrate is an antibiotic medication used to treat people in the event of bacterial infections. It is part of the larger antibiotic family known as the quinolones, and like its chemical relatives, this medication is considered effective at treating many different types of infection.

The chemical name of bromhexine hydrochloride is 2,4-dibromo-6-[[cyclohexyl(methyl)amino]methyl]aniline; hydrochloride and its empirical formula are $C_{14}H_{21}Br_2ClN_2$.[(3)]Bromhexine is a mucolytic agent used in the treatment of respiratory disorders associated with viscid or excessive mucus. A literature survey reveals that there is no HPLC method reported for the combination of Levofloxacin Hemihydrate [(4)]& Bromhexine HCl[(5)] [in] its fixed-dose combination. Therefore, an attempt has been made to develop a new HPLC method that is simple, rapid, reproducible, and economical for the estimation of Levofloxacin Hemihydrate &Bromhexine HCL in its combination. The proposed method was optimized and validated according to International Conference on Harmonization (ICH) guidelines [(6)].

MATERIALS AND METHODS

Chemicals and reagents:

An analytically Levofloxacin Hemihydrate & Bromhexine HCL Standard was procured from Central Drug Testing Laboratory, Mumbai with defined potency of 97.28 % (as is basis) and 99.32% (as is basis) respectively. Levofloxacin Hemihydrate & Bromhexine HCL oral suspension were received as a gift sample from Assistant Drugs Controller Office, Air Cargo, Mumbai. HPLC Grade acetonitrile from Ranken, potassium dihydrogen orthophosphate AR Grade from Rankem, ortho-phosphoric acid AR grade, HPLC Grade methanol from Ranken and water used during the analysis were of Mill Q grade and filter papers used for filtration were of 0.45µm nylon-66 filters.

Instrumentation:

LAB INDIA UV-VIS Spectrophotometer 3000$^+$ connected to a computer loaded with software UV Win 5 spectrophotometer was used for all the spectrophotometric measurements. HPLC method was developed on Perkin Elmer using software Perkin Elmer Chromatography Data System with LC instrument control.

Solubility profile:

Solubility of levofloxacin hemihydrate: The solubility study is carried out according to the Indian Pharmacopeia method. Levofloxacin hemihydrate is soluble in water, in methanol, in ethanol (95%); very slightly soluble in acetone, in acetonitrile, in chloroform, and practically insoluble in ether.

Solubility of Bromhexine HCl: Solubility of Bromhexine HCl according to IP 2018, is freely soluble in methanol; slightly soluble in ethanol (95%), and practically insoluble in acetone, chloroform, dichloromethane, and ether.

Selection of Diluents:

On the basis of the Molecular structure and chemical nature of Levofloxacin Hemihydrate & Bromhexine HCL, Methanol was selected as a diluent for the preparation of standard and sample solutions.

Selection of detection wavelength of Levofloxacin Hemihydrate & Bromhexine HCl:

Levofloxacin Hemihydrate standard solution of 200µg/ml was prepared by taking about 25.0mg of Levofloxacin Hemihydrate to a volumetric flask and the volume was made up to 25ml by water. Further 2ml of the above solution was diluted to a 10ml volumetric flask with water. Similarly, a standard solution of Bromhexine HCl15µg/ml was prepared by taking about 10.0mg of Bromhexine HClto volumetric flask, and the volume was made up to 200ml by water. Further 3ml of the above solution was diluted to a 10ml volumetric flask with water. Both the solution was scanned in the range of 200-400nm individually and the absorbance maximum of Levofloxacin Hemihydrate & Bromhexine HClwas found at 254 nm after overlaying.

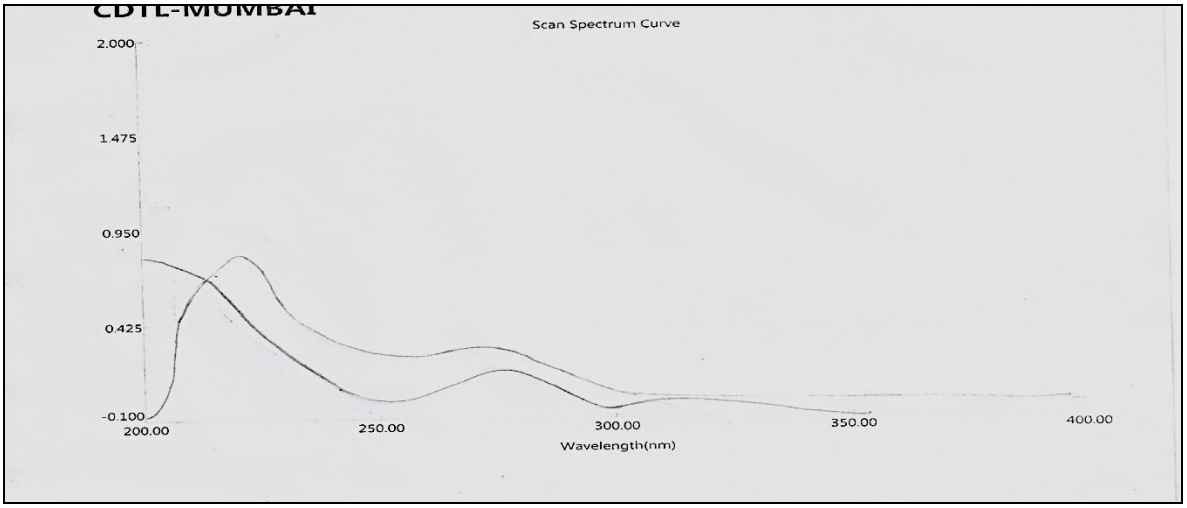

Figure No.3-Levofloxacin Hemihydrate & Bromhexine HCl UV Spectrum

Preparation of standard drug solution

Around 25mg Levofloxacin Hemihydrate and 10.0mg Bromhexine HCL were weighed on an analytical balance and dissolved in 10ml volumetric flasks respectively using diluent. From the above stock solution 2ml of Levofloxacin Hemihydrate (1000µg/ml) and 3ml of Bromhexine HCL (50 µg/ml) were withdrawn and diluted to 10ml volumetric flask with diluent. The final concentration was 200µg/ml and 15µg/ml of Levofloxacin Hemihydrate & Bromhexine HCL, which was treated as a final working standard solution.

Preparation of sample solution:

Around 1mg of Levofloxacin Hemihydrate & Bromhexine HCl was added to a 50ml volumetric flask. It was dissolved by sonicating in sufficient diluent, then both the stock solutions of the test were filtered using a syringe filter (0.45µm). Then, 1ml from Levofloxacin Hemihydrate & Bromhexine HClstock solution (20µg/ml) was diluted to 10ml with diluent. The final concentration of 200µg/ml and 15µg/ml of Levofloxacin Hemihydrate & Bromhexine HCl, which was treated as 100% target concentration.

Method optimization:

The chemical structure of Bromhexine HCL reveals that it is a strong base and highly polar compound having free solubility in water. Similarly, Levofloxacin Hemihydrate is a weakly basic and polar in nature having sparing solubility in water. Considering the nature of both the drug molecules initial trials were done on the C18 column with different buffers (potassium dihydrogen phosphate and ammonium phosphate), mobile phase ratio of the

aqueous and organic phase, but there were only changes in retention of Bromhexine HCL and no improvement in retention of Levofloxacin Hemihydrate in the column. For better retention of Bromhexine HCL Thermo Scientific Synchronise C18 column with dimensions 150 x, 4.6 x 5µ was used along with different concentrations of potassium dihydrogen phosphate buffer and its pH. Good peak shape with acceptable system suitability parameters was obtained with a mobile phase consisting of a mixture of 25mM potassium dihydrogen orthophosphate buffer (pH 3.0 with O-phosphoric acid) and acetonitrile in the ratio of 35:65 v/v. The flow rate was kept at 1 ml/min and UV detection wavelength of 254 nm and column oven temperature was maintained at 45˚C.

VALIDATION OF METHOD:

The developed RP-HPLC method of Levofloxacin Hemihydrate & Bromhexine HCL was validated as per ICH Q2 (R1) guidelines for parameters such as specificity, linearity, accuracy, and limit of detection, the limit of quantification, robustness.

Specificity

Specificity is the ability to access unequivocally the analyte in the presence of components that may be expected to be present. Blank, standard drug solution of Levofloxacin Hemihydrate & Bromhexine HCl(200µg/ml and 15µg/ml) and of Levofloxacin Hemihydrate & Bromhexine HCl(200µg/ml and 15µg/ml) were injected and their chromatograms were recorded. Chromatograms are shown in Figure no.4.1(a), (b), (c) show that there is no interference of excipient peak at the retention time of Levofloxacin Hemihydrate & Bromhexine HCL peak.

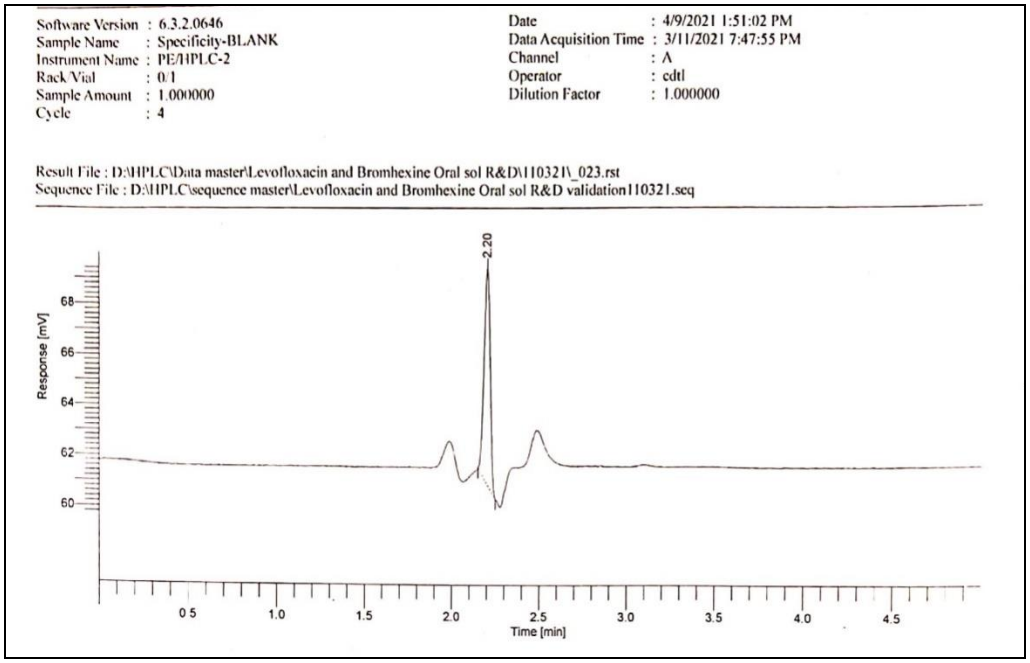

Figure No.4.1 (a)–Chromatogram of specificity of Levofloxacin Hemihydrate & Bromhexine HCl blank solution

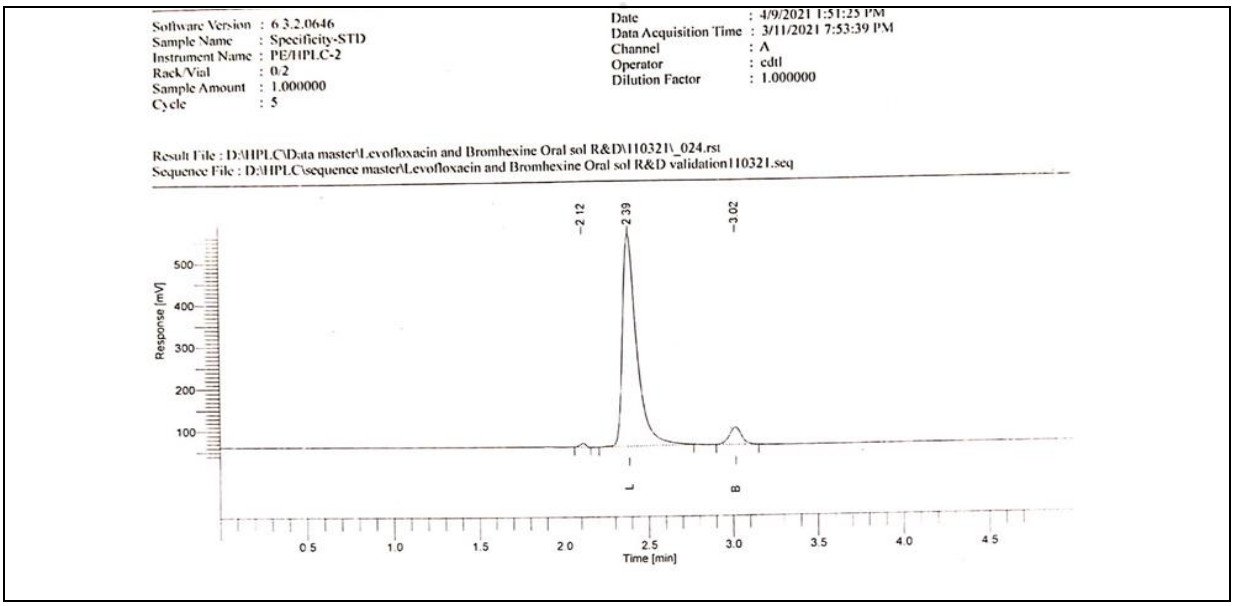

Figure No. 4.1 (b)–Chromatogram of specificity of Levofloxacin Hemihydrate & Bromhexine HCl standard mixture solution

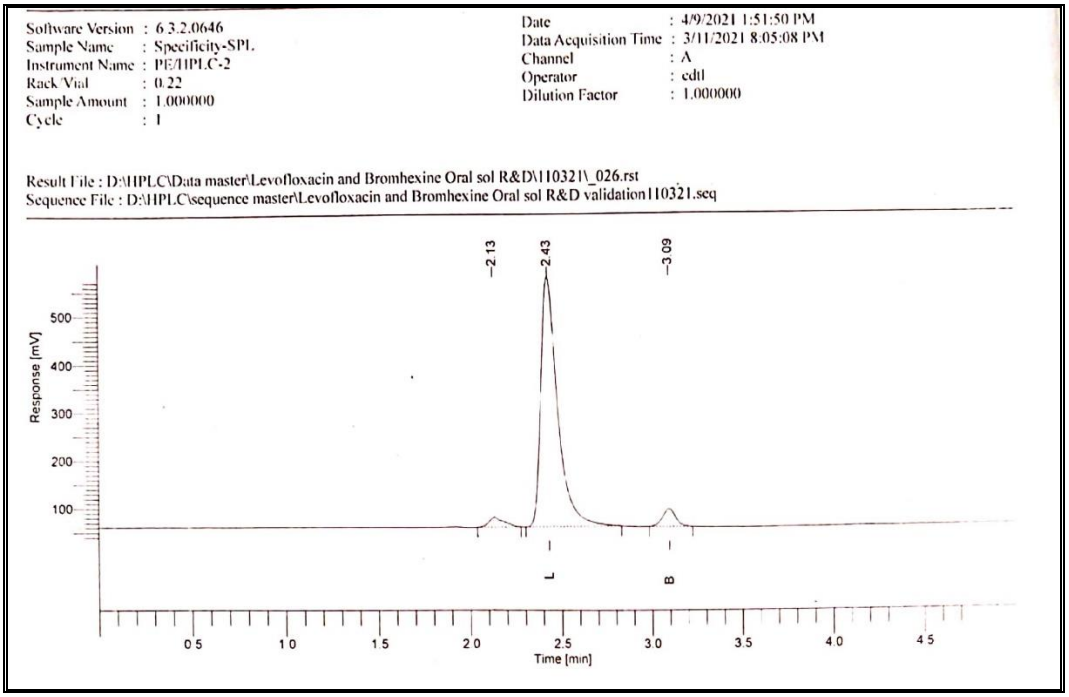

Figure No. 4.1 (c)-Chromatogram of specificity of Levofloxacin Hemihydrate & Bromhexine HCL sample solution

Linearity and Range

The linear response of the standard was determined over the range of 50-400µg/ml and 3.75-30µg/ml. The linear calibration plot was constructed by analyzing the concentration over the selected range versus the peak area of the standard solution and their results are mentioned inTable no.1.Regression coefficients were found to be 0.9995 and 0.9995 for the linearity response respectively from the calibration curve shown in Figure no.3.2(a) and 3.2(b)respectively.

Table No. 1-Result of linearity data of Levofloxacin Hemihydrate & Bromhexine HCl

Linearity level	Levofloxacin Hemihydrate		Bromhexine HCl	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	50	793031.5	3.75	46816
2	100	1465268	7.5	99355.5
3	150	2289990	11.25	149024
4	200	2976231	15	201916
5	250	3762629	18.75	256140.5
6	350	5318935	26.25	359369
7	400	6139069	30	402304

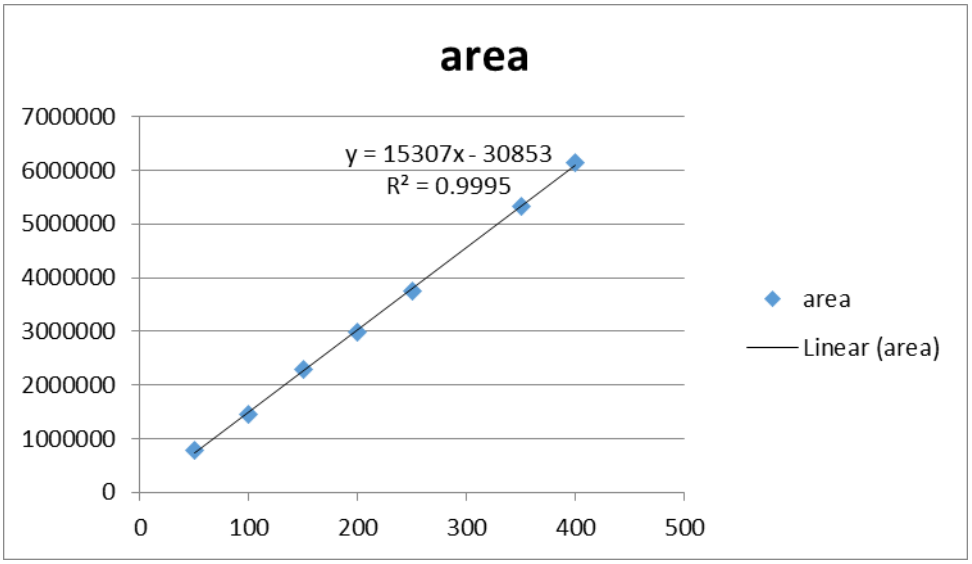

Figure No. 5.1 (a)-Calibration curve for Levofloxacin Hemihydrate

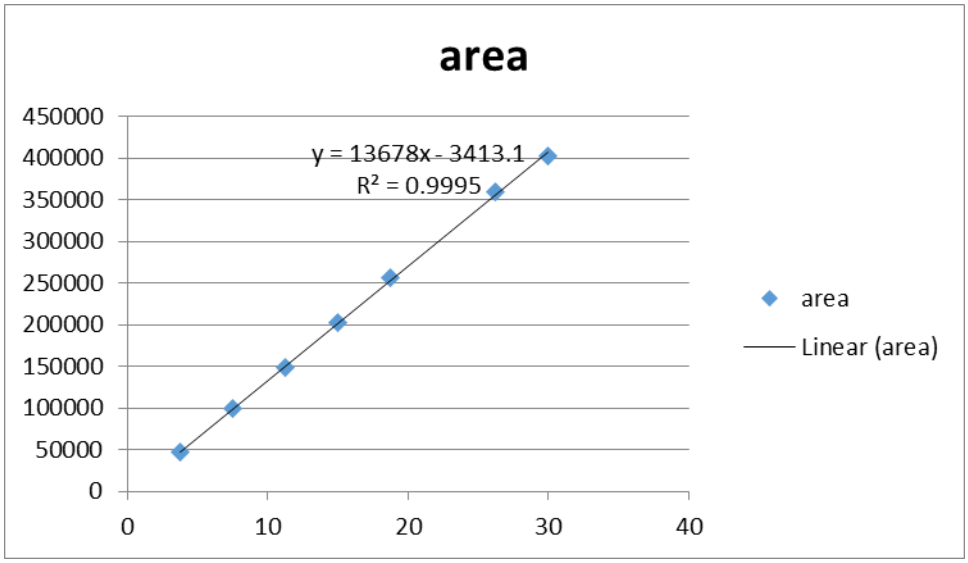

Figure No. 5.2 (b)-Calibration curve for Bromhexine HCl

Accuracy (Standard Addition method):

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the estimated value. The accuracy method was established by the standard addition method at three different levels 110%, 120%, and 130% it was determined by recovery of Levofloxacin Hemihydrate & Bromhexine HCl (known quantity) added to the test solution. The % mean recovery at all three levels was found to be between limits 98.0-102.0% for both Levofloxacin Hemihydrate & Bromhexine HCl. The mean %RSD values for Levofloxacin Hemihydrate & Bromhexine HCl were found to be 0.42 and 0.23 respectively mentioned in Table no. 2.

Table No. 2: Accuracy of levofloxacin Hemihydrate & Bromhexine HCl

% Level	Amount Spiked (ppm)		Amount Recovered (mg)		Recovery		% Recovery		Mean % Recovery		S.D.		%R.S.D.	
	levo	brom	levo	brom	Levo	brom	levo	brom	levo	brom	levo	brom	levo	brom
110	220	16.5	112.08	8.23	112.08	8.23	101.89	99.8	101.62	100.59	0.24	0.69	0.24	0.68
	220	16.5	111.56	8.34	111.56	8.34	101.42	101.04						
	220	16.5	111.71	8.33	111.71	8.33	101.55	100.94						
120	240	18	120.7	8.93	120.7	8.93	101.7	101.09	100.19	99.84	1.46	1.08	1.46	1.08
	240	18	118.54	8.93	118.54	8.93	100.08	99.19						
	240	18	122.05	9.1	122.05	9.1	98.78	99.24						
130	260	19.5	130.82	9.93	130.82	9.93	100.62	101.81	101.17	100.19	0.49	1.41	0.48	1.40
	260	19.5	131.74	9.74	131.74	9.74	101.33	99.29						0.68
	260	19.5	132.04	9.7	132.04	9.7	101.56	99.47						
								Mean	100.99	100.21	0.73	1.06	0.73	1.06
								Limit	98.0-102.0%		N.M.T 2.0 %		N.M.T 2.0 %	

Precision:

Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time.

System Precision: Six replicate injections of working standard solution of Levofloxacin Hemihydrate & Bromhexine HCl were injected. The %RSD calculated was found to be 1.07% &1.62% mentioned in Table no. 3.

Table No. 3: System precision of Levofloxacin Hemihydrate & Bromhexine HCl

Injection no.	Area		Limit
	Levofloxacin Hemihydrate	**Bromhexine HCl**	
1	2958680	197682	NMT 2%
2	2898471	193213	
3	2874359	201177	
4	2924421	193818	
5	2910489	193656	
6	2879651	193989	
Mean	2907679	195589.2	
± S.D.	31208.56	3183.856	
% RS.D.	1.073315	1.627828	

Method Precision: Six test solutions of Levofloxacin Hemihydrate & Bromhexine HCl were injected. The %RSD calculated was found to be 0.94%& 1.75%mentioned in Table No. 4.

Table No. 4: Method precision of Levofloxacin Hemihydrate & Bromhexine HCl

Injection no.	Area		Limit
	Levofloxacin Hemihydrate	**Bromhexine HCL**	
1	2834758	174293	NMT 2%
2	2853187	177397	
3	2811531	177366	
4	2848008	172950	
5	2887335	179165	
6	2821254	171119	
Mean	2842679	175381.7	
± S.D.	26925.62	3084.843	
% RS.D.	0.947192	1.758931	

Intermediate Precision: Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

Interday Precision: The study was carried out by injecting freshly prepared standard and test solutions on three different days and by three different analysts mentioned in Table no.5.

Table No. 5: Interday precision of Levofloxacin Hemihydrate & Bromhexine HCl

Sr.No.	Analyst A/ Day 1		Analyst B/ Day 2	
	Levo	Brom	Levo	Brom
1	101.48	100.04	99.4	101.34
2	100.31	100.04	100.45	98.46
3	99.53	99.73	100.64	98.06
4	99.52	100.38	99.84	99.86
5	98.95	99.73	100.16	99.02
6	101.65	99.52	101.99	99.01
Average	100.24	99.90667	100.4133	99.29167
S.D.	1.115096	0.307094	0.889757	1.173071
%R.S.D.	1.112427	0.307381	0.886094	1.18144

Intraday Precision: The study was carried out by injecting freshly prepared standard and test solution at three different time intervals i.e. at 10 am, 1 pm, and 4pmmentioned in Table no.6.

Table No. 6: Intraday precision of Levofloxacin Hemihydrate & Bromhexine HCl

Sr.No.	10.00 AM		1.00 PM		4.00 PM	
	levo	brom	levo	brom	levo	brom
1	98.48	98.09	98.06	99.57	99.5	99.02
2	101.83	101.03	99.6	101.34	100.89	98.8
3	101.9	99.54	99.42	98.8	100.64	98.06
4	101.76	98.31	101	99.11	99.84	99.86
5	99.67	101.13	100.9	101.69	100.45	98.46
Average	100.728	99.62	99.796	100.102	100.264	98.84
S.D.	1.567313	1.113226	1.21057	1.324451	0.576914	0.67587
%R.S.D.	1.555985	1.448731	1.213045	1.323101	0.575395	0.683802

Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. The method was established by a deliberate change in detection wavelength by ±2 nm, flow rate by ± 0.5 ml, change in the temperature by ± 2°C, and the ratio of mobile phase by ± 5 ml. It gives an indication of the reliability of the developed method during normal usage. The reproducible results were obtained which proves that method is robust as shown in Table no 7.

Table No. 7: Result of robustness studies of Levofloxacin Hemihydrate & Bromhexine HCl

Parameter	Change in Parameter		% Estimation		Mean		S.D.		% R.S.D.	
			levo	brom	levo	brom	levo	brom	levo	brom
Wavelength (nm)	252		101.3	98.6	100.75	99.53	0.56	0.88	0.55	0.89
	254		100.7	99.6						
	256		100.2	100.4						
Flow (ml/min)	0.6		101.5	99.2	101.10	99.71	0.39	0.59	0.38	0.39
	0.8		100.7	99.6						
	1		101.1	100.3						
Temperature (°C)	43		99.9	99.9	100.30	99.33	0.40	0.76	0.40	0.77
	45		100.7	99.6						
	47		100.2	98.5						
Mobile Phase Ratio	A	B								
[10mM Potassium dihydrogen phosphate(A): Methanol (B)]	40	60	101.5	98.4	100.97	99.24	0.44	0.74	0.43	0.75
	35	65	100.7	99.6						
	30	70	100.7	99.7						

System suitability test:

System suitability testing is an integral part of method development it was carried out as per ICH guidelines. The HPLC system used for analysis must pass the system suitability limits

before sample analysis can commence. A blank preparation (single injection) and standard preparation (six replicates) at the working concentration (200µg/ml of Levofloxacin Hemihydrate and 15µg/ml of Bromhexine HCl) were injected and the chromatograms were recorded and were assessed for different parameters such as retention time, peak area, no. of theoretical plates, tailing factor. Results of system suitability parameters are mentioned in Table no. 8.

Table No. 8: System Suitability of Levofloxacin Hemihydrate & Bromhexine HCl

Sr. No.	Peak Area		Retention Time		Theoretical Plates		Tailing Factor		Resolution
	levo	brom	levo	brom	levo	brom	levo	brom	levo/brom
1	2958680	197682	2.368	2.995	4235.63	7986.98	1.6	0.931	4.484
2	2923561	203358	2.399	3.015	4334.6	7402.06	1.581	0.917	4.31
3	2922193	202115	2.385	3.011	4315.06	7985.98	1.603	0.935	4.468
4	2918550	200447	2.377	2.999	4234.16	7984.06	1.593	0.92	4.435
5	2872502	204458	2.369	2.986	4406.72	7781.65	1.586	0.931	4.44
6	2993782	206150	2.386	3.013	4305.11	8188.48	1.601	0.951	4.496
Average	2931545	202368.3	2.380667	3.003167	4305.213	7888.202	1.594	0.930833	4.438833
S.D.	41026.58	3012.128	0.011776	0.011635			0.008944	0.012106	
%R.S.D.	1.399487	1.488438	0.494638	0.387415			0.561121	1.300606	
Limits	NMT 2.0%		NMT 1.0%		NLT 2000		NMT 2.0		NLT 2.0

RESULTS AND DISCUSSION:

High-pressure liquid chromatography (HPLC) is one of the most reliable and essential analytical tools for assessing the drug product. It has the ability to separate, identify, and quantify the compounds that are present in any sample. Therefore, a simple, accurate, precise, sensitive, and novel isocratic reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed for the estimation of levofloxacin Hemihydrate & Bromhexine HCL in in a combination. It can be used both for qualitative as well as quantitative analysis. After optimizing the method, with consideration of system suitability parameters, the method finalized was given below Table no. 9.

Table No. 9: Optimised RP-HPLC method for Levofloxacin Hemihydrate & Bromhexine HCl

COLUMN	Thermoscientific synchronise (150 x 4.6 x 5µ)
WAVELENGTH	254 nm
FLOW RATE	0.8 ml/min
INJECTION VOLUME	10µl
COLUMN OVEN	45
RUN TIME	10
SOLVENT MIXTURE	Methanol
MOBILE PHASE	25mM Potassium dihydrogen phosphate (pH-3.0 with Orthophosphoric acid): Acetonitrile [35:65]
DETECTOR	UV-VIS Variable wavelength

In the case of specificity, no interference was observed from blank (diluent) at the retention time of Levofloxacin Hemihydrate & Bromhexine HCL peak. Therefore, the developed RP-HPLC method for the determination of Levofloxacin Hemihydrate & Bromhexine HCL in combination is specific. The results of the linearity study of Levofloxacin Hemihydrate& Bromhexine HCL standard solution over the concentration range of 50-400µg/ml and 3.75-30µg/ml respectively and showed a linear response with a correlation coefficient of 0.9995 and regression equation y = 15307x – 30853for Levofloxacin Hemihydrate and correlation coefficient 0.9995 and regression equation y = 13678x - 3413.1 for Bromhexine HCL.

Accuracy was determined in terms of percent recovery studies at three different levels i.e., 110%, 120%, and 130%. The % mean recovery at all three levels was found to be between limits 98.0-102.0% for both Levofloxacin Hemihydrate & Bromhexine HCL. Average %RSD values for Levofloxacin Hemihydrate & Bromhexine HCL were found to be 0.42 and 0.23respectively.

In the repeatability method for system precision, replicate injections (n=6) of Levofloxacin Hemihydrate & Bromhexine HCL standard solution were injected and % RSD was found to be1.07 and 1.62 respectively. For method precision, 6 replicate injections of test solution

were injected %RSD was found to be 0.94 and 1.75 of Levofloxacin Hemihydrate &Bromhexine HCL respectively. This indicates that the method developed is highly precise.

The intermediate precision study (ruggedness) was ascertained on the basis of intraday and interday data obtained by analyzing Levofloxacin Hemihydrate & Bromhexine HCL in combination by HPLC method. Intraday precision was found to be reproducible with mean values of 100.79% and 100.20% and has an average % RSD less than 2 i.e. 0.39 and 0.53 for Levofloxacin Hemihydrate & Bromhexine HCL respectively. Interday precision was also found to be reproducible with mean values of 100.24% and 99.90% and has an average % RSD less than 2 i.e.1.11 and 0.37 for Levofloxacin Hemihydrate & Bromhexine HCL respectively. This indicates the ruggedness of the method. In the case of robustness, it was performed by changing various parameters such as wavelength, flow, temperature, and the ratio of mobile phase, and it was observed that the method was robust through the validation report, it can also be concluded that the system is suitable for analysis.

CONCLUSION

A simple and RP-HPLC chromatography method was developed for the determination of Levofloxacin Hemihydrate & Bromhexine HCl simultaneously and validated as per ICH guidelines. The result of validation studies proved that the proposed method was also accurate, precise, specific, robust, and sensitive. It possessed significant linearity, high efficiency, resolution, and no interference from the excipients. This method can be applied for qualitative and quantitative determination.

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