Analytical Method Developement and Validation of Beclomethasone, Clotrimazole, Ofloxacin, Lidocaine by RP-HPLC in Combination Dosage Form.

ABSTRACT

A Rapid and Precise method (in accordance with ICH guidelines) is required for the quantitative simultaneous determination of drugs in a combined pharmaceutical dosage form. The pursuit of desired quality is a continual challenge for pharmaceutical industries, necessitating a meticulous approach known as validation. Sensitive and specific RP-HPLC method involving UV detection was carried for determination and quantification of various drug in combination dosage form. The aim is " Analytical Method Developement and Validation of Beclomethasone, Clotrimazole, Ofloxacin, Lidocaine by RP-HPLC In Combination Dosage Form " as per ICH guidelines. The developed method was validated for various parameters as per ICH guidelines like system suitability, specificity, linearity, system precision, method precision, accuracy, ruggedness and robustness. The separation method was carried out by using a mobile phase A consisting of Potassium dihydrogen phosphate buffer (0.05M): Methanol 45:55 v/v) pH 3.5 ± 0.1 and Mobile phase B comprises Acetonitrile in the ratio of 50:50 v/v. The deduction was carried out by using UV detector at 300nm. The column was Agilent C 18 (250X4.6mm) 5µ. The flow rate was selected as 1.5 ml/min. The retention time of Ofloxacin, Lidocaine, Beclomethasone, and Clotrimazole, was found to be 5.67, 9.44, 18.29, and 21.85 respectively

Keywords: Beclomethasone, Clotrimazole, Lidocaine, Ofloxacin, and % RSD

1. INTRODUCTION

Lidocaine, Clotrimazole, Beclometasone, Ofloxacin are a combination of four medicines. Lidocaine is a local anesthetic which works by blocking pain signals from the nerves to brain to decrease pain sensationin the ear[1-2]. Clotrimazole is an antifungal which stops the growth of fungi [3-4] while Ofloxacin is an antibiotic that prevents bacterial cells from dividing and repairing, This treats your ear infection [5-6]. Beclometasone is a steroid. It blocks the production of certain chemical messengers (prostaglandins) that make the ear red, swollen and itchy [7- 8].

Ear Drop is a medicine used in the treatment of various types of ear infections. It prevents the growth of microorganisms that cause infection in the ears [9-10]. It also relieves the symptoms of inflammation such as pain, itchiness, and irritation [11-12]. The combination dosage form ear drops containing beclomethasone, clotrimazole, ofloxacin, and lignocaine offers a potent therapeutic option for treating various ear infections and inflammations.[13-14] Beclomethasone, a corticosteroid, provides anti-inflammatory action; clotrimazole, an antifungal agent, combats fungal infections; ofloxacin, a broad-spectrum antibiotic, targets bacterial infections; and lignocaine, a local anesthetic, alleviates pain and discomfort [15-16].To ensure the efficacy, safety, and quality of these ear drops, it is imperative to develop a robust and reliable analytical method for their quantification [17-18]. Reverse-phase high-performance liquid chromatography (RP-HPLC) stands as a preferred technique due to its versatility, sensitivity, and specificity in analyzing multi-component formulations [19-20]. The development and validation of an RP-HPLC method for the simultaneous determination of beclomethasone, clotrimazole, ofloxacin, and lignocaine in ear drops pose unique challenges, including

the need for optimal chromatographic conditions to achieve baseline separation of all compounds, as well as ensuring specificity in the presence of excipients or potential impurities [21-22]. This study aims to address these challenges by systematically developing and validating an RP-HPLC method tailored to the specific requirements of the combination ear drop formulation [23-24]. Key parameters such as specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and system suitability will be thoroughly evaluated according to international guidelines, including those established by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [15-24].

Beclomethasone dipropionate is a second-generation synthetic corticosteroid and diester of beclomethasone, which is structurally similar to dexamethasone. Beclomethasone dipropionate is used in various inflammatory conditions (Sweetman, 2009) [25]. Ofloxacin is a synthetic fluoroquinolone antibacterial agent that inhibits the supercoiling activity of bacterial DNA gyrase, halting DNA replication. Ofloxacin is an antibacterial agent used for the treatment of bacterial infections in many parts of the body, including the respiratory tract, kidney, skin, soft tissue, and urinary tract [26]. Clotrimazole is a broad-spectrum antimycotic or antifungal agent. Clotrimazole's antimycotic properties were discovered in the late 1960s. Clotrimazole falls under the imidazole category of azole antifungals, possessing broad-spectrum antimycotic activity [27]. Lidocaine is a local anesthetic agent commonly used for local and topical anesthesia, but it also has antiarrhythmic and analgesic uses and can be used as an adjunct to tracheal intubation. It is a tertiary amine and is a class Ib antiarrhythmic agent [28]. The validated RP-HPLC method will serve as a valuable tool for routine quality control analysis, enabling accurate and reliable quantification of the active pharmaceutical ingredients in the ear drops. Furthermore, it will contribute to ensuring compliance with regulatory standards and safeguarding patient safety and therapeutic efficacy (Snyder et al., 2010) [29].

2. MATERIAL AND METHODS

2.1 Instrumentation and Analytical Conditions

The HPLC analyses were carried out on an Agilent 1200 system (Santa Clara, CA), composed of a quaternary pump, autosampler, and DAD. Agilent HP ChemStation Software was used for data acquisition and analysis. Separations were performed on Agilent C18 column (250 × 4.6 mm id, 5 µm particle size) from Merck at 30°C. UV detection was at 290 nm for Beclomethasone and 300 nm for clotrimazole, ofloxacin, and lignocaine. Mobile phase A was a mixture of Buffer Potassium dihydrogen phosphate : Methonol ; [45:55 v/v] and mobile phase B was acetonitrile. Gradient elution was carried out as follows: 100% mobile phase A was first held for 5 min, then mobile phase B was raised up to 52% in 7 min, mobile phase B was held at this level until 12 min, and at 12.01 min, mobile phase A was switched back to 100% until 17 min (re-equilibration). The flow rate was 1.5 mL/min, and the injection volume was 20 µL.

2.2 Preparation of standard stock solution:

2.2.1 Beclomethasone:

Weigh and transfer accurately 1 mg of Beclomethasone working standard into a 100ml clean and dry volumetric flask and make up with 100ml of diluent and sonicate to dissolve.

2.2.2 Clotrimazole:

Weigh and transfer accurately 40 mg of Clotrimazole working standard into a 100ml clean and dry volumetric flask and make up with 100ml of diluent and sonicate to dissolve.

2.2.3 Ofloxacin:

Weigh and transfer accurately 12 mg of Ofloxacin working standard into a 100ml clean and dry volumetric flask and make up with 100ml of diluent and sonicate to dissolve.

2.2.4 Lidocaine:

Weigh and transfer accurately 80 mg of Lidocaine working standard into a 100ml clean and dry volumetric flask and make up with 100ml of diluent and sonicate to dissolve.

2.3 Sample Solution

Prepare the sample test solution equivalent to the Lidocaine 800 ppm, ofloxacin 120 ppm, Clotrimazole 400 ppm, Beclomethasone 10 ppm, in 100ml volumetric flak, added 100ml of diluents phase sonicated for 30 minutes. Make up the volume with diluents. Mixed well and filtered through 0.45µ nylon filter paper discarded first few ml of the filtrate.

2.4 EXPERIMENT

2.4.1 Optimization of mobile phase:

Separation of both the drugs was tried using the following combination of mobile phases. The table 1 gives the details of the same.

Table 1 Optimization of Mobile Phase

Sr No.	Mobile phase	Ratio (v/v)	Eluation of Peak
1	Buffer: Acetonitrile	**30 : 70**	Not Proper Separation
2	Buffer: Acetonitrile	**50 : 50**	Not Proper Separation
3	Methanol: Acetonitrile	**45 : 55**	Separation of Peak
4	Buffer: Methanol	**20:80**	Separation of Peak
5	[Buffer : Methonol; 45:55 v/v] : Acetonitrile	**50:50**	**Good Separation**

Out of 5 trials the **5th trial was selected for further studies** because when compared to other trails. 4th trial was found less in retention time due to the ratio of organic solvent in mobile phase.

2.4.2 Selection of Wavelength

Solution of Beclomethasone, Clotrimazole, Ofloxacin and Lidocaine were scanned in the UV region and spectrum was recorded (200-400nm). The solvent used was[Buffer : Methonol : 45:55 v/v] : Acetonitrile Adjust pH with potassium dihydrogen phosphate buffer in ratio of 50:50. It was seen that 300nm as shown in figure 1 All compounds have very good absorbance, which can be used for the estimation of compounds by HPLC.

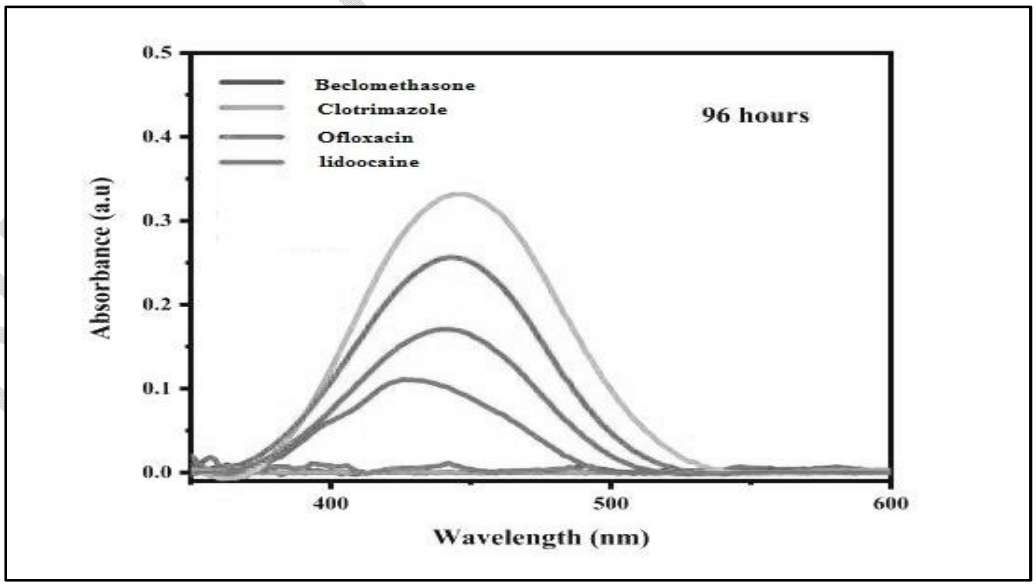


Figure 1 Overlay UV spectrum of Beclomethasone, Clotrimazole, Ofloxacin and Lidocaine showing selection of wavelength detection.

2.4.3 Optimized Conditions

The following optimized parameters were used as a final method for the simultaneous estimation of Beclomethasone, Clotrimazole, Ofloxacin and Lidocaine.

2.4.3.1 Instrument

Column	Agilent C18 (250x4.6mm) 5µ
Column Oven Temperature	30° C
Wave length	300 nm
Flow rate	1.5 ml/min
Injection Volume	20 µl
Runtime	30 minutes
Mode of Operation	Reverse phase

2.4.3.2 Mobile Phase

Solvent A	(Potassium dihydrogen phosphate Buffer& Methanol 45:55 v/v)
Solvent B	(Acetonitrile-50 v/v)
Solvent ratio	50 v/v of Solvent A: B

2.4.4 Method Validation

The method was validated according to International Conference on Harmonization Guidance for Industry Q2 (R1) Validation of Analytical Procedures: Text and Methodology (13).

3. RESULTS AND DISCUSSION

3.1 SYSTEM SUITABILITY

System suitability of the method was performed by calculating the parameters namely, resolution and number of theoretical plates on the 6 replicate injection of standard solution into HPLC system and calculates. The %RSD for 6 replicate injections should not more than 2.0%. The system suitability parameters and % RSD for peak areas for 6 replicate injections of standard solution was found to be within limits presented in table 2.

Table 2 Result System suitability

Inj No.	OFLOXACIN		LIDOCAINE		BECLOMETHASONE		CLOTRIMAZOLE	
	RT	Area Response	RT	Area Response	RT	Area Response	RT	Area Response
1	5.67	12517517	9..44	14893246	18.29	1014107	21.85	278518
2	5.64	12474563	9..46	14896344	18.31	996936	21.89	276745
3	5.62	12524563	9..43	14517517	18.27	1002914	21.91	278976

4	5.68	12465631	9..45	14861819	18.36	1021217	21.86	278570
5	5.63	12615668	9..49	14813093	18.25	1002291	21.83	271971
6	5.68	12535678	9.52	14897540	18.26	1007492	21.88	275611
%RSD	**0.99**		**1.02**		**0.89**		**0.96**	

3.2 SPECIFICITY

The specificity of the RP-HPLC method was demonstrated by the distinct retention times(shown in table 3) for each active pharmaceutical ingredient: Beclomethasone at 18.29 minutes, Clotrimazole at 21.85 minutes, Lidocaine at 9.44 minutes, and Ofloxacin at 5.67 minutes. There was no interference from the diluent or mobile phase at 300 nm, confirming the method's specificity for the quantification of these compounds.see figure 2.

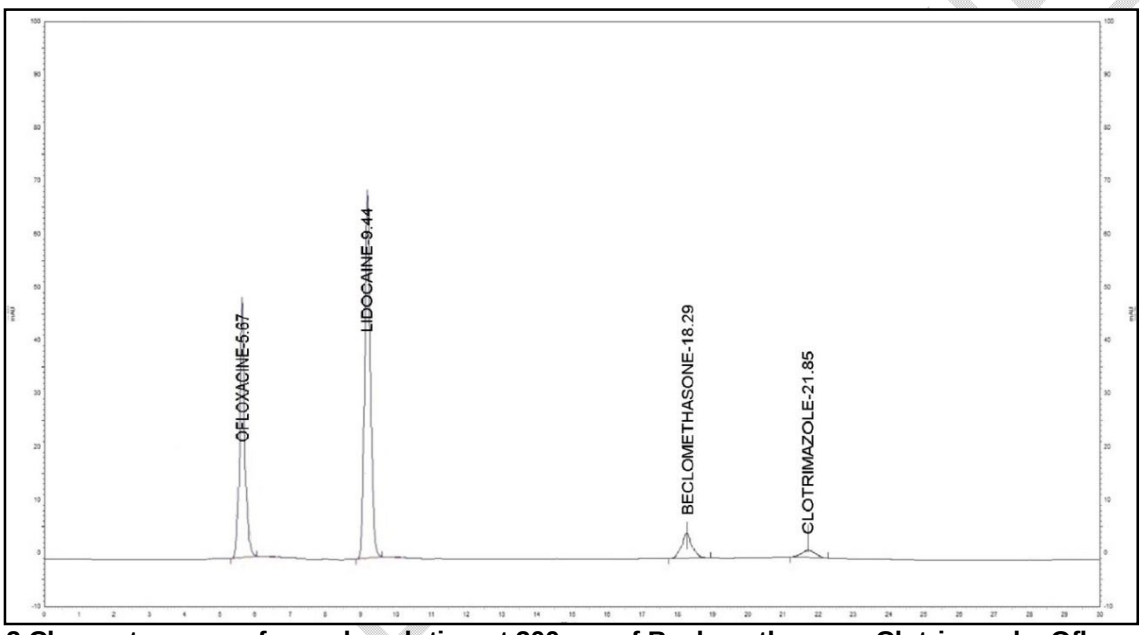
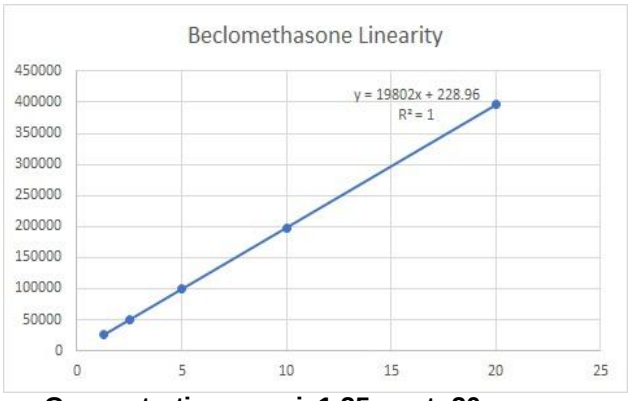


Figure 2 Chromatograms of sample solution at 300 nm of Beclomethasone, Clotrimazole, Ofloxacin and Lidocaine

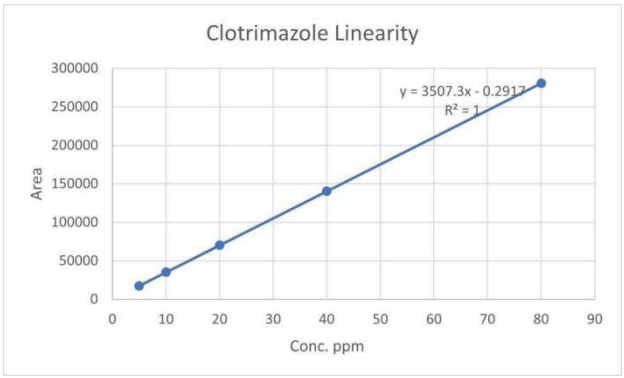
3.3 LINEARITY

The linearity of the RP-HPLC method was assessed by preparing a series of standard solutions at varying concentrations. Appropriate aliquots of the four-drug combination (1.25, 2.5, 5, 10, and 20 ml) were pipette from the stock solution into a series of 100 ml volumetric flasks and diluted to volume with the makeup phase. (figure 4 Each concentration was then injected (20 ul) into the HPLC system under the optimized chromatographic conditions. The concentration range for each drug, along with the corresponding area of response and correlation coefficient, is provided in Table 4.

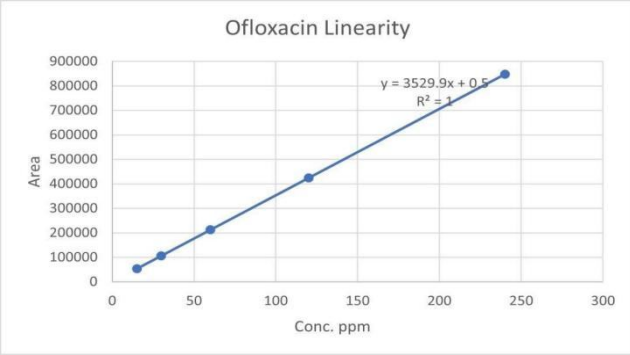
These results demonstrate that the method exhibits excellent linearity across the tested concentration ranges for all four compounds. The correlation coefficients for Beclomethasone, Clotrimazole, and Ofloxacin were exactly 1.0, indicating a perfect linear relationship between concentration and response. For Lidocaine, the correlation coefficient was slightly above 1.0, which may indicate an extremely high degree of linearity within the tested range



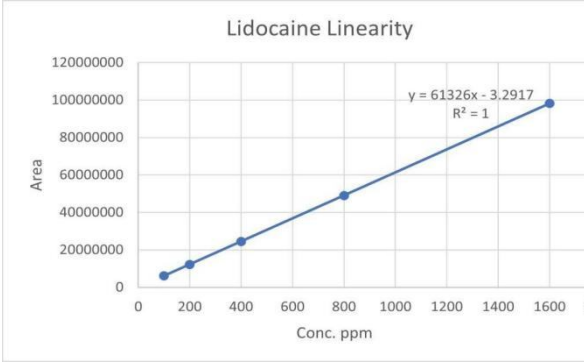
Concentrationrangeis1.25ppmto20ppm



Concentrationrangeforis5ppm**to80ppm**



Concentrationrangeforis15ppmto240ppm



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Concentrationrangeforis 100ppmto1600ppm

Figure 3 ; The linearity graphs for Beclomethasone, Clotrimazole, Ofloxacin, and Lidocaine These figures illustrate the direct proportionality between the concentration of the analytes and their corresponding responses, validating the method's suitability for quantitative analysis.

Table 3 Overview of the linearity data and Statistics.

Drug	Linearity level	Concentration (PPM)	Area response	Correlation coefficient
Beclomethasone	1	1.25	25131	1.0
	2	2.5	49543	
	3	5	99086	
	4	10	198172	
	5	20	395102	

Clotrimazole	**1**	**05**	**17539**	**1.0**
	2	**10**	**35072**	
	3	**20**	**70145**	
	4	**40**	**140290**	
	5	**80**	**280580**	
Ofloxacin	**1**	**15**	**52951**	**1.0**
	2	**30**	**105894**	
	3	**60**	**211792**	
	4	**120**	**423583**	
	5	**240**	**847169**	
Lidocaine	**1**	**100**	**6132563**	**1.01**
	2	**200**	**12265100**	
	3	**400**	**24530262**	
	4	**800**	**49060504**	
	5	**1600**	**98120991**	

3.4 ROBUSTNESS

The robustness of the method was determined by carrying out experiment at different flow rate and variation in mobile phase composition.Result of the Robustness at different variable parameter given in table 5.Results for actual mobile phase composition (50:50 Acetonitrile: Methanol & phosphate buffer) has been considered from accuracy standard.

Table 4 Result of the Robustness at different variable parameter

Sr. No	Name	Flow Rate (ml/min)	System Suitablity Result		Change in composition Mobile Phase	System Suitablity Result	
			Plate Count	Sp Tailling		Plate Count	Sp Tailling
1	**Ofloxacin**	0.5	4236	1.48	2 % less	4239	1.43
		1	4239	1.48	Actual	4242	1.43
		1.5	4963	1.56	2% More	4966	1.51
2	**Lidocaine**	0.5	5156	1.57	2 % less	5159	1.52
		1	5158	1.57	Actual	5161	1.52
		1.5	5363	1.66	2% More	5367	1.61

3	**Beclomethasone**	0.5	3369	1.31	2 % less	3371	1.26
		1	2983	1.27	Actual	2987	1.22
		1.5	3102	1.29	2% More	3104	1.24
4	**Clotrimazole**	0.5	2156	1.20	2 % less	2159	1.19
		1	2346	1.19	Actual	2349	1.17
		1.5	2349	1.19	2% More	2353	1.19

The % RSD of retention time and asymmetry were within limits for variation (+ 2 %) in composition of mobile phase. Hence the method was found to be robust.

3.5 PRECISION

Standard solutions containing (0.1, 0.2, 0.4 µg/mL) of Beclomethasone, (0.4, 0.8, 1.6 µg/mL) of Clotrimazole, (1.2, 2.4, 3.6µg/mL) of Ofloxacine and (8, 16, 32 µg/mL) of Lidocaine were analyzed three times on the same day for the determination of intra-day precision and on three different days for the determination of inter-day precision. % RSD values for intra-day and inter-day precision are represented in table 7.

Table 5 Repeatability Data For Ofloxacin, Lidocaine, Beclomethasone, Clotrimazole

Ofloxacin			**Lidocaine**			**Beclomethasone**			**Clotrimazole**		
Conc (ug/ml)	PeakArea	% RSD	Conc (ug/ml)	PeakArea	% RSD	Conc (ug/ml)	Peak Area	% RSD	Conc (ug/ml)	Peak Area	% RSD
	12517517			14893246			1014107			278518	
	12474563			14896344			996936			276745	
	12524563			14517517			1002914			278976	
60	12465631	0.99	**400**	14861819	1.02	**5**	1021217	0.89	**20**	278570	
	12615668			14813093			1002291			271971	0.96
	12535678			14897540			1007492			275611	

Table 6 Day And Inter-Day Precision For Ofloxacin, Lidocaine, Beclomethasone, Clotrimazole

Beclomethasone		**Clotrimazole**		**Ofloxacin**		**Lidocaine**	
Conc (ug/ml)	Mean=S.D	Conc (ug/ml)	MeanS.D	Conc (ug/ml)	Mean S.D	Conc (ug/ml)	Mean S.D
Intra-Day Precision							
0.1	1014110 =15.9	0.4	136985=1.92	1.2	6258745=3.92	8	7416623=16.21

0.2	2031562 =37.3	0.8	278518=8.56	2.4	12517517=3.71	16	14833246=22.23
0.4	3953565=47.21	1.6	560053=32.91	3.6	24936245=3.77	32	28555657=35.76
Inter-Day Precision							
0.1	1014113 =26.9	0.4	136981=1.92	8.2	6258753=3.02	8	7416623=26.41
0.2	2031568 =48.3	0.8	278526=8.56	14.3	12517524=9.24	16	14833246=62.31
0.4	3953559=68.21	1.6	560062=32.91	28.9	24936249=11.78	32	28555657=95.69

3.6 ACCURACY

Accuracy was calculated at three different levels in terms of % recovery by spiking known amount of standard solution (80%, 100% and 120%) to the solution of a synthetic laboratory mixture of Beclomethasone, Clotrimazole, Ofloxacin and Lidocaine. % mean recovery of each drug is given in table 8, 9, 10 and 11 respectively.

Table 7 Accuracy in Terms of % Recovery for Beclomethasone

Conc. Level(%)	Sample amount (ug/mL)	Amount of Standard Added (ug/mL)	Beclomethasone		
			Amount Recovered (ug/mL)	% Recovery	% Mean Recovery
80%	5	4	4.02	100.5	100.5
	5	4	4.06	101.5	
	5	4	3.98	99.5	
100%	5	5	4.99	99.8	100.6
	5	5	4.98	99.6	
	5	5	5.12	102.4	
120%	5	6	6.01	100.16	100.55
	5	6	6.13	101.83	
	5	6	5.98	99.66	

Table 8 Accuracy in Terms of % Recovery for Clotrimazole

		Amount of	Clotrimazole

Conc. Level (%)	Sample amount (ug/mL)	Standard Added (ug/mL)	Amount Recovered (ug/mL)	% Recovery	% Mean Recovery
80%	20	16	16.24	101.21	100.70
	20	16	15.94	99.62	
	20	16	16.26	101.29	
100%	20	20	20.19	100.74	99.67
	20	20	19.78	99.12	
	20	20	19.79	99.17	
120%	20	24	23.57	98.57	98.84
	20	24	23.88	99.60	
	20	24	23.51	98.36	

Table 9 Accuracy in Terms of % Recovery for Ofloxacin

Conc. Level (%)	Sample amount (ug/mL)	Amount of Standard Added (ug/mL)	Ofloxacin		
			Amount Recovered (ug/mL)	% Recovery	% Mean Recovery
80%	60	48	48.12	100.25	100.24
	60	48	47.87	99.72	
	60	48	48.36	100.75	
100%	60	60	60.23	100.38	100.02
	60	60	60.01	100.01	
	60	60	59.81	99.68	
120%	60	72	72.11	100.15	99.96
	60	72	72.09	100.12	
	60	72	71.73	99.62	

Table 10 Accuracy in Terms of % Recovery for Lidocaine

Conc. Level (%)	Sample amount (ug/mL)	Amount of Standard Added (ug/mL)	Lidocaine		
			Amount Recovered (ug/mL)	% Recovery	% Mean Recovery
80%	400	320	320.56	100.17	100.02
	400	320	319.81	99.94	
	400	320	319.88	99.96	
100%	400	400	400.04	100.01	100.08
	400	400	401.00	100.25	
	400	400	399.98	99.99	
120%	400	480	480.96	100.20	100.05
	400	480	480.01	100.00	
	400	480	479.82	99.96	

4. CONCLUSION

UV Detector and Agilent C18 (250x4.6mm) 5µ column, injection of 20µl is injected and eluted with the mobile phase of acetonitrile and Potassium Dihydrogen phosphate buffer with pH 3.5 & Methanol (45:55 v/v) in the ratio 50:50 which was pumped at a flow rate of 1.5ml at 300nm. The peak of within limits was found well separated within 30 min. The developed method was validated for various parameters as per ICH guidelines like system suitability, specificity, linearity, system precision, method precision, accuracy, ruggedness and robustness.

In conclusion, the developed RP-HPLC method proved to be reliable, accurate, and sensitive for the quantitative analysis of beclomethasone, clotrimazole, ofloxacin, and lignocaine in combination ear drops. This validated method can be effectively utilized for routine quality control analysis and batch release of pharmaceutical formulations, ensuring product safety and efficacy.

ABBREVIATIONS

µL - Microliter (volume)
mL - Milliliter (volume)
nm - Nanometer (wavelength)
µg - Microgram (mass)
mg - Milligram (mass)
g - Gram (mass)
% - Percentage (concentration or proportion)
min - Minute (time)
18. °C - Degrees Celsius (temperature)
19. AU - Absorbance Units (absorbance measurement)

HPLC - High-Performance Liquid Chromatography
UV - Ultraviolet (Detector)
MS - Mass Spectrometry (Detector)
RT - Retention Time
RP-HPLC - Reverse Phase High-Performance Liquid Chromatography
LOD - Limit of Detection
LOQ - Limit of Quantitation
API - Active Pharmaceutical Ingredient
C18 - Octadecylsilane (common type of stationary phase))
%R - Percentage Recovery
ICH Q2(R1) - International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Guideline Q2(R1): Validation of Analytical Procedures: Text and Methodology
%RSD - Percent Relative Standard Deviation
NMT - Not More Than

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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