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Validated RP-H-P-L-C Method for the Simultaneous Determination of Betamethasone and Ofloxacin in Bulk and Pharmaceutical Formulations

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ABSTRACT

The immobile fluid utilized for separation is a Kromosil® C18 H-P-L-C Column with a size of particle which is 5 µm & a pore size of 100 Å, measuring 250 mm in distance end to end & 4.6 mm in diameter (internal). The MP consists of KH_2PO_4 & Acetonitrile in a 55:45 ratio, maintained at a Flow Rate (FR) of 1.0 mL/min, with a maximum wave vector of 278 nm & a temp set at 30.0°C. The average retention times recorded for Betamethasone & Ofloxacin were 2.331 mins & 3.312 mins respectively. The percentage recovery was recorded at 100.09% for Betamethasone & 100.62% for Ofloxacin.

Keywords: Betamethasone, Ofloxacin, RP-H-P-L-C.

INTRODUCTION

Microbial diseases have a profound effect on public health. Infections can occur in any part of the body, with either the bacteria themselves or the body's response leading to illness. Humans can acquire bacteria from various sources, including living organisms, atmosphere, water & food. The primary modes of transmission for microbial infections include direct contact, aerosolized droplets, vectors, & contaminated vehicles. Preventive measures significantly decrease morbidity & mortality rates. These measures encompass water disinfection, vaccination of humans & animals, promoting safer sexual practices, & enhancing personal hygiene. The rise of antibiotic resistance in bacteria is an emerging concern that necessitates prudent antibiotic usage. Bacteria are omnipresent & play a crucial role in maintaining our ecosystem. Only a small fraction of bacteria are responsible for infections & diseases, which have a significant impact on public health. Antimicrobial agents with antimicrobial properties are increasingly accessible, facilitating the treatment of microbial infections compared to viral ones. However, the growing issue of microbial resistance to these antimicrobials poses a serious threat, potentially more severe than diseases caused by viruses & parasites. Ocular microbial infections are among the primary contributors to morbidity & vision impairment. The rise of antibiotic resistance (AMR) is progressively undermining the effectiveness of early antibiotic interventions, which are essential for preserving eyesight.[1,2]

In this instance, we evaluated the cause of microbial infections in the eyes observed at Massachusetts Eye & Ear & investigated the molecular epidemiology & antimicrobial resistance profiles of recent isolates.

In primary care, eye infections are a frequent presenting issue. Conjunctivitis, "red eye," & "corneal ulcer/keratitis" were the top five issues. The practitioner should diagnose the patient as soon as possible & begin the necessary treatment to guarantee a positive visual outcome for the patient. While infections of the cornea or inside the eye pose a major risk to vision, conjunctivitis usually poses no such threat & should be treated immediately by referral to an ophthalmologist.[3-5]

In the present state, as antimicrobial & steroid combinations that are frequently used to treat microbial infections, the effectiveness & safety of the combination of Ofloxacin & betamethasone are researched & developed.[6] According to certain claims, it lessens middle ear mucosal oedema & ocular region redness from infection, which stops microbes from colonizing the allergic site & lessens the sensitivity & allergies of the anti-biotic component inside the liquid drops.[7]

Analyte Background

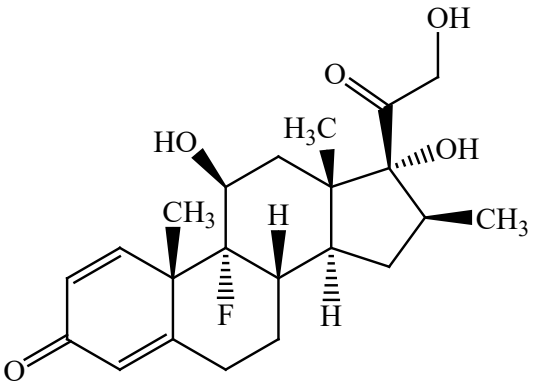
The combination of two medications is called betamethasone & ofloxacin. A steroid called betamethasone inhibits the synthesis of prostaglandins, which are chemical messengers that induce swelling, redness, & itching. An antibiotic called ofloxacin kills bacteria by stopping their ability to divide & repair. This takes care of your infection.[8,9]

Betamethasone

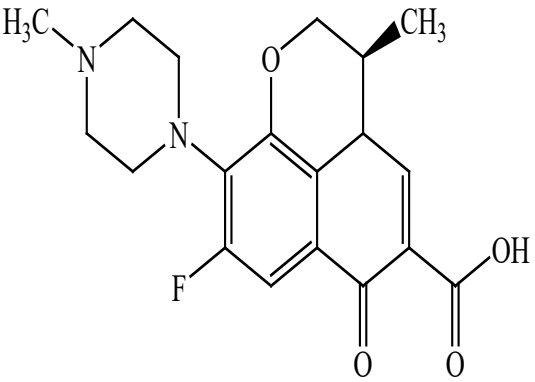
Betamethasone, a corticosteroid which is long-acting, possesses anti-inflammatory & immunosuppressive properties. It can be administered via injection to address various medical settings, including autoimmune disorders, & can also be applied topically to manage inflammatory skin issues such as eczema. Betamethasone demonstrates significant glucocorticoid effects while exhibiting minimal mineralocorticoid activity. For the treatment of plaque psoriasis, it can be used topically in conjunction with a vitamin D analog like calcipotriene. Furthermore, betamethasone functions through both genomic & nongenomic pathways. The genomic pathway is characterized by glucocorticoids activating glucocorticoid receptors, leading to flowing effects that promote the transcription of genes which are anti-inflammatory, including tyrosine amino transferase (T-A-T), phosphoenolpyruvate carboxykinase (P-E-P-C-K), & the IL-1 receptor antagonist.[10,11]

Ofloxacin

Treating microbial infections in the kidney, skin, soft tissues, respiratory tract, & urinary tract, among other regions of the body, is done with this antimicrobial drug. The antimicrobial agent known as fluoroquinolones is a synthetic compound that prevents microbial DNA gyrase from supercoiling, hence stopping DNA replication.[12,13]



Structure of Betamethasone Depiction: 1 (Source: Drawn in Chemdraw)



Structure of Ofloxacin Depiction: 2 (Source: Drawn in Chemdraw)

H-P-L-C is a highly effective analytical technique for the separation & quantification of drugs. Currently, there are no reported RP-H-P-L-C methods in the literature for the estimation of Betamethasone & Ofloxacin, either in combination/ individually dosage forms.[8] A review of existing literature indicates the presence of more

economical methods; therefore, it is essential to develop & validate a straightforward, cost-effective stability-indicating simultaneous estimation of Betamethasone & Ofloxacin using RP-H-P-L-C in pharmaceutical dosage forms, in accordance with I-C-H rules (Q2 specifications).[14,15]

MATERIALS & REAGENTS

1. Betamethasone & Ofloxacin were sourced as pure substances from Symbiotec Pharma Lab.
2. From Rankem Chemical Division H-P-L-C-grade methanol & acetonitrile were obtained in India.
3. From Rankem Potassium-hydrogen-phosphate was also acquired, India.
4. (Rankem, India) Pure milli-Q water was utilized, filtered through 0.45µ Millipore filters.

Chromatographic Settings & Instrumentation

The WATERS H-P-L-C model (System 2695), equipped with a photodiode array detector, utilized for method authenticate & development, featuring an automated sample injector. Kromosil C18 (4.6 mm ID x 250 mm x 5µ) served as the separation medium. The Mobile Phase (MP) consisted of 0.01N KH_2PO_4 as phase A & Acetonitrile as phase B in a 55:45 ratio. The study was performed in isocratic mode at a FR of 1.0 mL/min, with an injection vol 10 µL. The column was maintained at a temp of 30 degree centigrade, & the total run time was 8 minute. Data acquisition occurred at a detection wave vector of 278 nm using Empower 2 software.

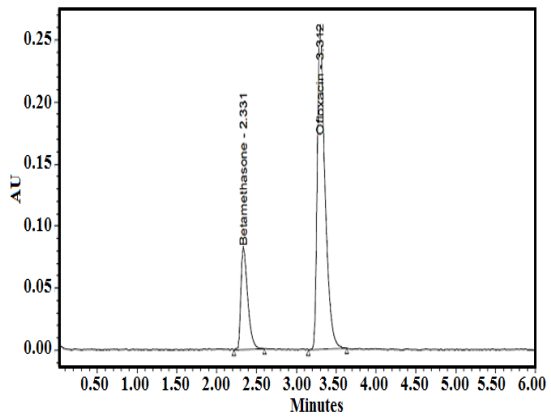


Fig. 1. Enhanced Chromatogram of Betamethasone & Ofloxacin I Source: attached is the chromatogram file in folder labeled Figure 1

Sr. No	Peak Name	RT	Area	USP Tailing	USP Resolution	USP Plate Count
1	Betamethasone	2.331	98765	1.5		3595.0
2	Ofloxacin	3.312	286467	1.5	5.6	5584.1

Solutions Preparation

Diluent: (50:50v/v) ratio of Water & Acetonitrile.

Buffer Preparation

KH_2PO_4 Buffer: Precisely measure 0.1% of ortho phosphoric acid in a 1000 mL flask which is volumetric, add approximately 900 mL of milli-Q water, degas by sonication, & then adjust the vol with water to achieve a pH of 2.8.

Preparation of Standard solution: Accurately Weighed & transferred 2mg of Betamethasone & 6 mg of Ofloxacin working Standards into 25 mL clean dry flask which is volumetrics, add 10 mL of diluent, sonicated for 10 mins & make up to the final vol with diluents. (80µg/mL Betamethasone & 240 µg/mL Ofloxacin).

Standard Working Solution

1 mL of standard concentrated solution was transferred to 10 mL flask which is volumetric & made up with diluent. (8 µg/mL Betamethasone & 24 µg/mL Ofloxacin).

Preparation of Sample Concentrated solution: A total of 0.01 mg w/v of Betamethasone & 0.03% w/v of Ofloxacin was extracted from the sample container & transferred into a clean, dry 25 mL flask which is volumetric. Approximately 7 mL of diluent was added, & the mixture was sonicated until fully dissolved. The vol was then adjusted to the mark with the same solvent & ran through through a 0.45-micron injection filter using a syringe. Subsequently, 1 mL of the Concentrated solution was pipetted into a 10 mL flask which is volumetric & madeup to the mark with diluent, resulting in concentrations of 40 µg/mL for Betamethasone & 120 µg/mL for Ofloxacin.

Sample Working Solution

2 mL of standard concentrated solution was transferred to 10 mL flask which is volumetric & made up with diluent. (8 µg/mL Betamethasone & 24 µg/mL Ofloxacin).

Method Authenticate: The authenticate of the H-P-L-C method was conducted for the concurrent estimation of Betamethasone & Ofloxacin drug substances in accordance with I-C-H rules, to establish that the method is suitable for regular study. System suitability: The assessment of system suitability was conducted for each authenticate parameter by introducing a system suitability solution comprising Betamethasone at a concentration of 8 µg/mL & Ofloxacin at 24 µg/mL. The elution profile illustrating system suitability is presented in Fig. 2, with the corresponding values detailed in Table 1.

Specificity (Selectivity): The evaluation of interference in the enhanced method indicates that no interfering Max outs should be present in the blank & placebo samples at the retention times of these drugs. Consequently, this method is considered specific. A representative elution profile is illustrated in Fig. 3, & the experimental data is presented in Table 1.

Table 1: System suitability chart

Betamethasone			Ofloxacin			
(min) RT	TP	Tailing	(min) RT	TP	Tailing	RS
2.322	3454	1.50	3.235	5876	1.50	5.3
2.322	3659	1.53	3.240	5538	1.59	5.4
2.325	3376	1.53	3.244	5639	1.57	5.4
2.327	3450	1.54	3.249	5876	1.50	5.4
2.330	3410	1.50	3.256	5909	1.57	5.4
2.330	3390	1.50	3.260	5637	1.58	5.5

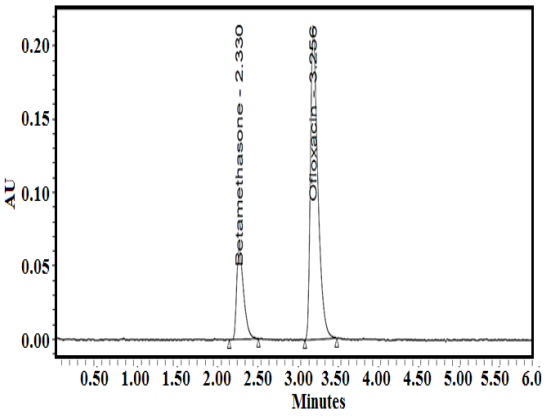


Fig. 2. System suitability Elution profile of Betamethasone & Ofloxacin I Source: attached is the chromatogram file in folder labeled Figure 2

Table 2: Specificity data

Sample name	(mins) Retention time
Betamethasone	2.325
Ofloxacin	3.352

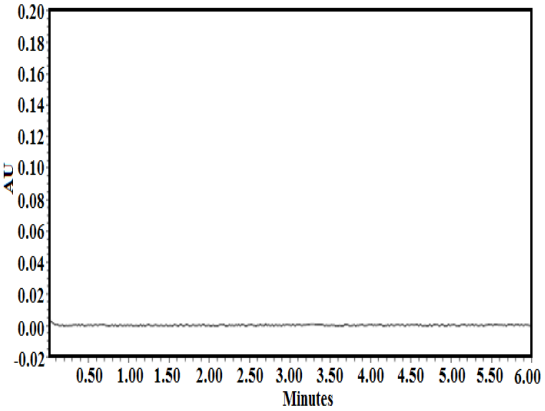


Fig. 3. Specificity & Overlay representation of H-P-L-C Elution profile of Betamethasone & Ofloxacin I Source: attached is the chromatogram file in folder labeled Figure 3

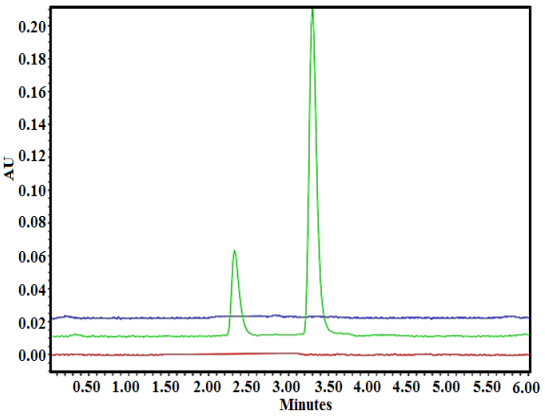


Fig. 4. Purity plots I Source: attached is the chromatogram file in folder labeled Figure 4

The elution profile presented above indicates that there was no interference detected in the blank & placebo solutions at the retention times of Betamethasone & Ofloxacin. All compounds are well separated with satisfactory resolution.

In order to assess the stability-indicating characteristics of the H-P-L-C method, samples of Betamethasone & Ofloxacin were subjected to stress settings including acid, base, oxidation, thermal exposure, light, & water. The resulting degraded samples were analyzed using a photodiode-array detector, & the Max out purity for both Betamethasone & Ofloxacin was confirmed. The settings for forced degradation are detailed in Table 3, while the corresponding outcomes are presented in Table 4.

The outcomes indicated that degradation Max outs were noted when the samples were subjected to acid exposure. The stress study revealed that none of the degradants co-eluted with the Max outs of the active drug that were formed.

Table 3: Forced degradation settings for Betamethasone & Ofloxacin

Stress condition	Solvent	Temp in degree Celsius	Exposed time in min
Acid	2N HCL	60°C	30
Base	2N NAOH	60°C	30
Oxidation	20% H_2O_2	60°C	30
Thermal	Diluent	105°C	360 h
Photolytic	Diluent	-	-
Hydrolytic	Water	60°C	

Table 4: Degradation profile outcomes

Degradation condition	Betamethasone% Undegraded	Ofloxacin% Undegraded
Acid	93.60	93.22
Base	94.52	96.68
Oxidation	98.80	97.74
Thermal	97.71	98.37
Photolytic	98.70	98.75
Hydrolytic	99.98	99.04

L-O-D & L-O-Q: The detection boundary indicates the lowest concentration of an analyte in a sample that can be recognized, even if it cannot be quantified. In contrast, the L-O-Q is the minimum concentration of an analyte that can be accurately & precisely measured using the specified method. The L-O-D values for Betamethasone & Ofloxacin are shown in Table 5, accompanied by the relevant elution profile depicted in Figure 5.

The L-O-Q values obtained for Betamethasone & Ofloxacin are listed in Table 5 & 6 corresponding representative elution profile is shown in Figure 6.

Table 5: Summary of L-O-D

Sample	Conc(µg/mL)	Max out area	S/N Ratio
Betamethasone	0.05	16185	8.2
Ofloxacin	0.05	59326	23.4

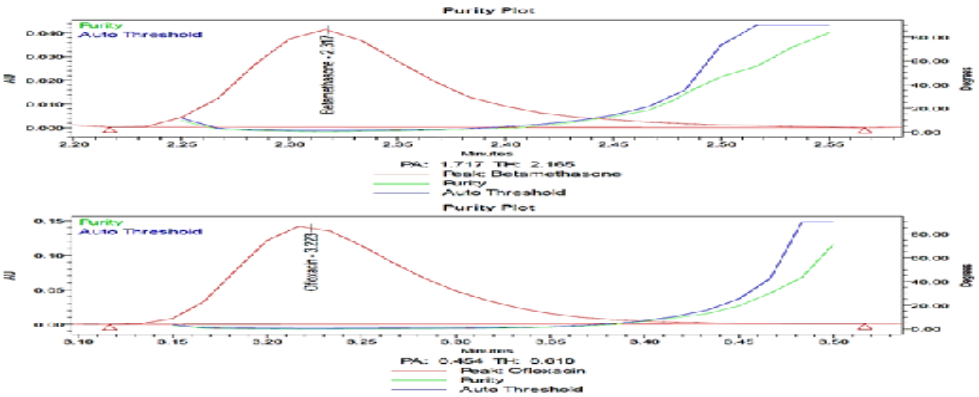


Fig. 5. Typical representation of H-P-L-C Elution profile of L-O-D Solution. Based on above outcomes for L-O-D, S/N ratio of each component was within the boundary I Source: attached is the chromatogram file in folder labeled Figure 8

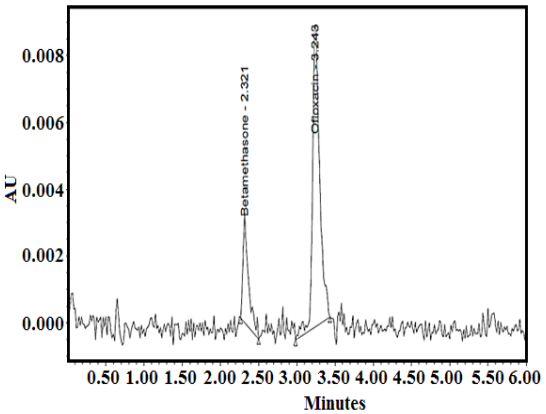


Fig. 6. Typical representation of H-P-L-C Elution profile of L-O-Q Solution. Based on above outcomes for L-O-D, S/N ratio of each component was within the boundary I Source: attached is the chromatogram file in folder labeled Figure 9

Table 6: Summary of L-O-Q

Sample	Conc(µg/mL)	Max out area	S/N Ratio
Betamethasone	0.16	47361	24.4
Ofloxacin	0.15	171324	68.9

Linearity: The method's linearity was established for Betamethasone & Ofloxacin by examining solutions that varied from 25% to 150% of the specified boundary (refer to Table 7). The correlation coefficient obtained for both Betamethasone & Ofloxacin was 0.999, which signifies a strong linear relationship (see Figure 7).

Assay data

Festive-D Eye/Ear Drops bearing the label claims Betamethasone 0.01% W/v, Ofloxacin 0.03%

w/v. Assay was performed with the above formulation. Average %Assay for Betamethasone & Ofloxacin obtained was 99.10 & 100.33% respectively. Assay data shown in Table 8.

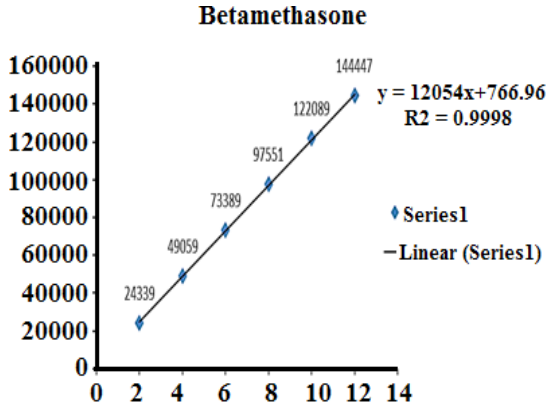


Fig. 7. Linearity plot of Betamethasone

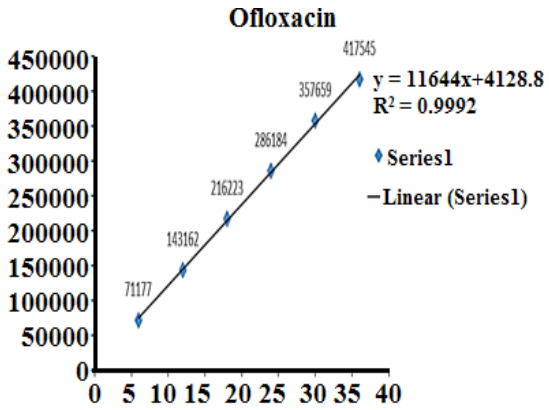


Fig. 8. Linearity plot of Ofloxacin

Table 7: Linearity data

	Betamethasone		Ofloxacin	
% Level	Conc(µg/mL	Area	Conc(µg/mL	Area
25%	2	24339	6	71177
50%	4	49059	12	143162
75%	6	73389	18	216223
100%	8	97551	24	286184
125%	10	122089	30	357659
150%	12	144447	36	417545

Table 8: Assay data of Betamethasone & Ofloxacin

Standard Area of Betamethasone	Standard Area of Betamethasone	% Assay of Betamethasone	Standard Area of Ofloxacin	Standard Area of Ofloxacin	% Assay of Ofloxacin
96587	96846	99.11	284456	287577	100.87
96787	96945	99.22	283646	284457	99.78
98765	96497	98.76	287556	286547	100.51
97654	96946	99.22	286467	287456	100.83
97654	96897	99.17	284578	287674	100.90
97645	96843	99.11	280457	282454	99.07
97515	96829	99.10	284527	286028	100.33
775.8	168.8	0.173	2461.2	2128.8	0.75
0.8	0.2	0.2	0.9	0.7	0.7

$$\%\ Assay = \frac{AT}{AS} \times \frac{WS}{25} \times \frac{1}{10} \times \frac{25}{1} \times \frac{10}{2} \times \frac{P}{100} \times \frac{FV}{L.C} \times 100$$

AT	Average Peak area of in test solution
AS	Mean peak area of in standard solution
WS	Weight of working standard taken in mg
P	Assay of working standard in % on dried basis
L.C	Label Claim
FV	Filled volume(1ml of a vail)

Preparation of Sample Concentrated solution: A sample containing 0.01 mg w/v of Betamethasone & 0.03% w/v of Ofloxacin was pipetted from the sample container into a clean,

dry 25 mL flask which is volumetric. Approximately 7 mL of diluent was added, & the mixture was sonicated until fully dissolved. The vol was then adjusted to the mark with the same solvent & filtered through a 0.45-micron injection filter using a syringe. Subsequently, 1 mL of the Concentrated solution was pipetted into a 10 mL flask which is volumetric & diluted to the mark with diluent. Additionally, 2 mL of the standard Concentrated solution was transferred to a 10 mL flask which is volumetric & brought to vol with diluent, resulting in concentrations of 8 µg/mL for Betamethasone & 24 µg/mL for Ofloxacin.

Accuracy: The accuracy of the method was assessed by utilizing solutions that included spiked samples of Betamethasone & Ofloxacin at concentrations of 50%, 100%, & 150% of the working strength. All solutions were prepared in triplicate & subsequently analyzed. The % recovery outcomes for each impurity are presented in Table 9.

Table 9: % Recovery data

%Level	%Recovery	
	Betamethasone	Ofloxacin
50% Level	100.73	100.95
	99.05	100.10
	100.71	99.37
100% Level	100.14	100.90
	99.94	100.86
	100.05	100.47
150% Level	99.72	100.93
	99.79	101.14
	100.71	100.88
Mean%	100.09	100.62

System accuracy: The system accuracy was performed by analyzing six replicate injections of working solution at 100% of the specified boundary with respect to the working strength of Betamethasone & Ofloxacin. Outcomes of Max out area are summarized in Table 10.

Table 10: System accuracy data

Injection	Betamethasone	Ofloxacin
1	96587	284456
2	96787	283646
3	98765	287556
4	97654	286467
5	97654	284578
6	97645	280457
Avg	97515	284527
Std dev	775.8	2461.2
% R-S-D	0.8	0.9

The %R-S-D for the Max out areas of Betamethasone & Ofloxacin obtained from six replicate injections of working solution was within the boundary.

Method Accuracy: A frequently used statistical term is the standard error of the mean of a population of observations. The standard error of the mean is calculated as the square root of the total of the squared deviations of each individual result from the mean, divided by the total number of outcomes minus one. The standard error of the mean, denoted as S, is expressed as follows:

$$S = \sqrt{\frac{\sum_{i=1}^{n}(x - x')^2}{(n-1)}}$$

The standard error of the mean shares the same units as the measured property. The square of the standard error of the mean is referred to as variance (S^2). This is defined as the standard error of the mean divided by the mean, represented as S/x. This value is occasionally multiplied by 100 to express it as a percentage, known as the percent relative standard error of the mean, which provides a more dependable indication of accuracy.

$$\% = \frac{SD}{Mean} \times 100$$

The accuracy of the method was assessed through the study of a sample consisting of Betamethasone & Ofloxacin, involving six separate sample preparations. The outcomes are compiled in Table 11.

Table 11: Method accuracy data

Injection	Betamethasone	Ofloxacin
1	96846	287577
2	96945	284457
3	96497	286547
4	96946	287456
5	96897	287674
6	96843	282454
Avg	96829	286028
Std dev	168.8	2128.8
%R-S-D	0.2	0.7

From the above outcomes, the %R-S-D of method accuracy study was within the boundary for Betamethasone & Ofloxacin.

Intermediate accuracy differs from repeatability in that it reflects the accuracy achieved within a single laboratory over an extended duration,

typically several months, & takes into account a wider range of variables than repeatability. Specifically, it includes variations such as different analysts, calibration Standards, reagent batches, columns, & spray needles. While these factors remain consistent within a single day, they are not s-Table over longer periods, thus exhibiting random behavior in the context of intermediate accuracy. As a result, since intermediate accuracy considers more influencing factors, its value, represented as standard error of the mean (as detailed in the following section), is greater than that of the repeatability standard error of the mean. The data collected is presented in Table 12.

Table 12: Intermediate accuracy data

Injection	Betamethasone	Ofloxacin
1	96857	281556
2	96876	284445
3	96476	288646
4	96164	284754
5	96456	286454
6	96086	283567
Avg	96486	284904
Std dev	333.0	2434.5
%R-S-D	0.3	0.9

From the above outcomes, the %R-S-D of method accuracy study was within the boundary for Betamethasone & Ofloxacin.

Robustness: The chromatographic parameters were intentionally modified to assess the robustness of the current method. To evaluate this robustness, a system suitability solution was prepared according to the established methodology & injected into the H-P-L-C under various altered settings. These settings included adjustments to the FR (±10%), column oven temp (±5°C), & MP composition (±10%) from the original method specifications. No significant changes were noted in the method's performance with respect to flow, temp, or MP, & the system suitability criteria were met as per the methodology. The outcomes regarding robustness are presented in Table 13.

Table 13: Robustness outcomes

Chromatographic condition	Betamethasone (R-S-D)	Ofloxacin (R-S-D)
Flow(-)	0.3	0.3
Flow(+)	0.4	0.5
Temp(-)	0.6	0.2
Temp(+)	0.2	0.4
m.p.(-)	0.5	0.3
m.p(+)	0.3	1.6

CONCLUSION

A straightforward, precise, & reliable method has been established for the concurrent assessment of analytes in Table formulations. The system suitability was evaluated by performing 6 injections of the standard, with outcomes consistently meeting the acceptance criteria (Boundary of <2). A linearity assessment was conducted across levels ranging from 25% to 150%, yielding an R^2 value of 0.999. Various authenticate parameters, including accuracy, accuracy, boundary of detection L-O-D, L-O-Q, & robustness, were confirmed to be within accept Table boundary. The percentage recovery was recorded at 100.09% for Betamethasone & 100.62% for Ofloxacin. This method is characterized by its simplicity, accuracy, sensitivity, rapidity, & cost-effectiveness, with a total runtime of fewer than 8 minute. It is practically applicable for the determination of assay in Table formulations.

ACKNOWLEDGEMENT

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Conflict of interest

No Conflict of Interest

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