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STABILITY INDICATING METHOD DEVELOPMENT FOR THE ESTIMATION OF DIFLUPREDNATE AND MOXIFLOXACIN IN EYE DROP FORMULATION BY HPLC

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ABSTRACT

A simple, rapid, precise, and stability-indicating reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous estimation of Difluprednate (DFP) and Moxifloxacin (MXF) in ophthalmic formulations. Chromatographic separation was achieved on a suitable C18 column using an optimized mobile phase under isocratic conditions, with detection at an appropriate UV wavelength. The developed method showed good linearity over the concentration ranges of 1–5 µg/ml for DFP and 5–25 µg/ml for MXF, with correlation coefficients (r^2) of 0.999 for both drugs. System suitability parameters were within acceptable limits, indicating satisfactory chromatographic performance. Accuracy studies demonstrated mean recoveries ranging from 97.94–99.04% for DFP and 98.20–98.41% for MXF. Precision studies showed %RSD values below 2%, confirming the repeatability and robustness of the method. The limits of detection and quantification were found to be 0.15 and 0.45 µg/ml for DFP and 0.10 and 0.35 µg/ml for MXF, respectively. The method was successfully applied to the assay of a marketed ophthalmic formulation. Forced degradation studies under acidic, alkaline, oxidative, and photolytic conditions confirmed the stability-indicating nature of the method, as degradation products were well separated from the analyte peaks. The validated method is suitable for routine quality control and stability studies of Difluprednate and Moxifloxacin in ophthalmic dosage forms.

Introduction

Difluprednate (a difluorinated prednisolone derivative) is a potent corticosteroid used topically in the management of ocular inflammation, while moxifloxacin is a fourth-generation fluoroquinolone used as a broad-spectrum antibacterial agent in ophthalmic preparations [1-2]. Co-formulation of a corticosteroid and a fluoroquinolone in eye drops is common to provide combined anti-inflammatory and anti-infective therapy following ocular surgery or severe inflammation; accurate assay and impurity profiling of both actives in such multi-component formulations is essential to ensure safety and efficacy [3].

Stability-indicating methods are analytical procedures that quantitatively determine the active pharmaceutical ingredients (APIs) in the presence of their degradation products, excipients and other potential impurities, and are required for shelf-life assignment and change control [4-5]. For ophthalmic products where pH, preservative interactions, light exposure and oxidants can affect both potency and safety validated stability-indicating liquid chromatographic assays are the preferred approach because of their selectivity and capability to separate and quantify related substances [6-7].

Regulatory guidance requires that analytical procedures intended for assay and impurity testing demonstrate specificity, accuracy, precision, linearity, range, robustness and limit of quantitation per ICH Q2(R1) [7], and that forced degradation (acid/base hydrolysis, oxidation, photolysis, and thermal stress) be performed to demonstrate the method is stability-indicating as part of ICH stability expectations. Therefore, development of a single RP-HPLC method capable of simultaneous quantification of difluprednate and moxifloxacin and separation of their degradation products simplifies routine quality control and stability studies, reduces analysis time and conserves sample.

Literature reports describe validated RP-HPLC and UPLC methods for individual estimation of difluprednate and moxifloxacin, as well as several approaches for simultaneous estimation in ophthalmic dosage forms [8-14]; however, published methods differ in stationary phases, mobile-phase composition (buffers, organic modifier ratios), detection wavelengths and gradients/isocratic modes indicating the need to optimize chromatographic conditions (column chemistry, pH, organic composition, flow and gradient) and to demonstrate forced-degradation separation for the specific commercial formulation under study. This work therefore aims to develop and validate a rapid, robust, stability-indicating RP-HPLC method for simultaneous assay of difluprednate and moxifloxacin in eye-drop formulation in compliance with ICH guidelines and to apply the method to forced degradation studies.

Material and Methods

Material

Difluprednate and Moxifloxacin reference standards were used as received. HPLC-grade acetonitrile, methanol, and water were employed for the preparation of the mobile

phase. Analytical reagents of AR grade were used throughout the study. A marketed ophthalmic formulation containing Difluprednate and Moxifloxacin was procured from the local market and used for method application and assay analysis.

Methods

Initially to estimate Difluprednate and Moxifloxacin in fix dosage form number of mobile phase in different ratio were tried. Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was Acetonitrile: Methanol in the ratio of 20:80v/v. The mobile phase was filtered through 0.45µ filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min.

Selection of Diluent

Diluent used for preparation of sample were compatible with mobile phase and no any significant affect retention and resolution of analyte. After various trials methanol was used as diluents.

Preparation of Stock Solution:

Accurately weighed 10 mg API of DFP and MXF was transferred into 10 ml volumetric flask separately and added 5ml of methanol as diluents, sonicated for 20 minutes and volume was made up to 10ml with methanol to get concentration of solution 1000µg/ml (Stock-A)

Preparation of Sub Stock Solution:

5 ml of solution was taken from stock-A of both the drug and transferred into 50ml volumetric flask separately and diluted up to 50 ml with diluent (methanol) to give concentration of 100µg/ml of DFP and MXF respectively (Stock-B).

Preparation of Different Solution

0.1ml, 0.2ml, 0.3ml, 0.4ml and 0.5ml of stock-B were taken separately in 10 ml volumetric flask and volume was made up to 10ml with (methanol). This gives the solutions of 1µg/ml, 2µg/ml, 3µg/ml, 4µg/ml and 5µg/ml, for DFP. In same manner 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml and 25µg/ml of MXF also prepared.

Linearity and Calibration Graph

To establish the linearity of analytical method, a series of dilution ranging from 1-5µg/ml for DFP and 5-25µg/ml for MXF were prepared. All the solution were filtered through 0.45µm membrane filter and injected, chromatograms were recorded at 254.0 nm and it was repeat for five times. A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

System suitability parameters

Separation variables were set and mobile phase was allowed to saturate the column at 1.00 ml/min. After complete saturation of column, six replicates of working standard of DFP 5µg/ml for DFP and 10µg/ml MXF was injected separately. Peak report and column performance report were recorded for all chromatogram.

Validation of developed Method [8]

Linearity

Linearity of analytical procedure is its ability (within a given range) to obtain test which are directly proportional to area of analyte in the sample. The calibration plot was contracted after analysis of five different concentrations (from 1 to 5 µg/ ml for DFP) and (5 to 25µg/ ml for (MXF) and areas for each concentration were recorded three times and mean area was calculated. The response ratio (response factor) was found by dividing the AUC with respective concentration.

Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present such as impurities, degradation products and matrix components.

Accuracy

Recovery studies were performed to calculate the accuracy of developed method to preanalysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed.

Precision

The stock solution was prepared. The precision are established in three differences:

Repeatability

The repeatability was performed for five replicate at five concentrations in linearity range 5, 10, 15, 20 and 25µg/ml for DFP and 1, 2, 3, 4 and 5µg/ml for MXF indicates the precision under the same operating condition over short interval time. Results of repeatability are reported in table.

Intermediate Precision

Day To Day Precision

Intermediate precision was also performed within laboratory variation on different days and different analyst in five replicate at five concentrations. Results of day to day intermediate precision for DFP and MXF reported in table.

Robustness

As per ICH norms, small but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of

mobile phase was change from Acetonitrile: Methanol (20:80 % v/v) to (15:85 % v/v). Results of robustness are reported in table.

Detection Limit and Quantitation Limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

Analysis of both the drug in Tablet Sample

The eye drop equivalent to 5mg of DFP and 0.5mg of MXF was transferred to 10 ml volumetric flask containing methanol. The solution was sonicated for 25 min and the final volume was made with mobile phase. The mixture was then filtered through a 0.45 µm filter. The stock solution was further diluted sufficiently with methanol to get sample solution of drug concentration of 10µg/mL DFP and 5µg/mL MXF respectively. The amounts of DFP and MXF in eye drop formulation were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with formulation. Results of tablet analysis are reported in table.

Forced degradation studies

In order to determine whether the method is stability indicating, forced degradation studies were conducted on drug powder and the analysis was carried out by HPLC with a U.V. detector. 20µl of each of forced degradation samples were injected.

Acid degradation:

50 mg of both the drug sample was taken into a 50 ml separate round bottom flask, 50 ml of 0.1 M HCl solution was added and contents were mixed well and kept for constant stirring for 8 h at 80ºC. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of drugs.

Alkaline hydrolysis:

50 mg of the drug sample was taken into a 50 ml separate round bottom flask, 50 ml of 0.1 M NaOH solution was added and contents were mixed well and kept for constant stirring for 8 h at 80ºC. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of drugs

Oxidative degradation:

50 mg of the drug sample was taken into a 50 ml separate round bottom flask, 50 ml of 3% hydrogen peroxide solution was added, and contents were mixed well and kept for constant stirring for 24 hr at room temperature. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC

and calculate the percentage degradation using calibration curve of drugs.

Thermal degradation:

50 mg of the drug sample was taken in to a petridish and kept in oven at 50ºC for 4 weeks. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of drugs.

Results and Discussion

A simple, precise, and stability-indicating RP-HPLC method was successfully developed and validated for the simultaneous estimation of Difluprednate (DFP) and Moxifloxacin (MXF) in ophthalmic formulations in accordance with ICH Q2(R1) guidelines. Both drugs were well resolved with distinct retention times, as depicted in Figure 1, confirming the suitability of the chromatographic conditions for simultaneous analysis.

Linearity was established over the concentration ranges of 1–5 µg/ml for DFP and 5–25 µg/ml for MXF**,** with excellent correlation coefficients ($r^2 = 0.999$) for both analytes (Table 1). The regression parameters indicated a strong linear relationship between concentration and peak area, demonstrating the reliability of the method for quantitative analysis.

System suitability parameters (Table 2) confirmed the adequacy of the chromatographic system. Theoretical plate counts were well above acceptable limits, tailing factors were close to unity, and retention time variability was minimal, indicating good column efficiency, peak symmetry, and reproducibility of the method.

The accuracy of the method was demonstrated through recovery studies at 80%, 100%, and 120% levels for both drugs (Table 3). Mean percentage recoveries ranged from 97.94–99.04% for DFP and 98.20–98.41% for MXF, with %RSD values below 2%, confirming the accuracy and absence of interference from formulation excipients.

Precision studies, including repeatability, day-to-day precision, analyst-to-analyst precision, and robustness, showed %RSD values well below the acceptable limit of 2% for both drugs (Table 4). These results indicate excellent precision, ruggedness, and robustness of the method under normal and slightly varied analytical conditions.

The sensitivity of the method was established by low values of LOD and LOQ **(**Table 5**),** indicating the capability of the method to detect and quantify both drugs at low concentration levels.

The applicability of the developed method was confirmed by assay of the marketed ophthalmic formulation (Table 6). The percentage assay values of 96.00% for DFP **and** 98.00% for MXF demonstrated that the method is suitable for routine quality control analysis.

Forced degradation studies under acidic, alkaline, oxidative, and photolytic stress conditions revealed that both DFP and MXF underwent measurable degradation, while the method effectively separated degradation products from the parent drug peaks (Tables 7 and 8). This confirms the stability-indicating nature of the developed method.

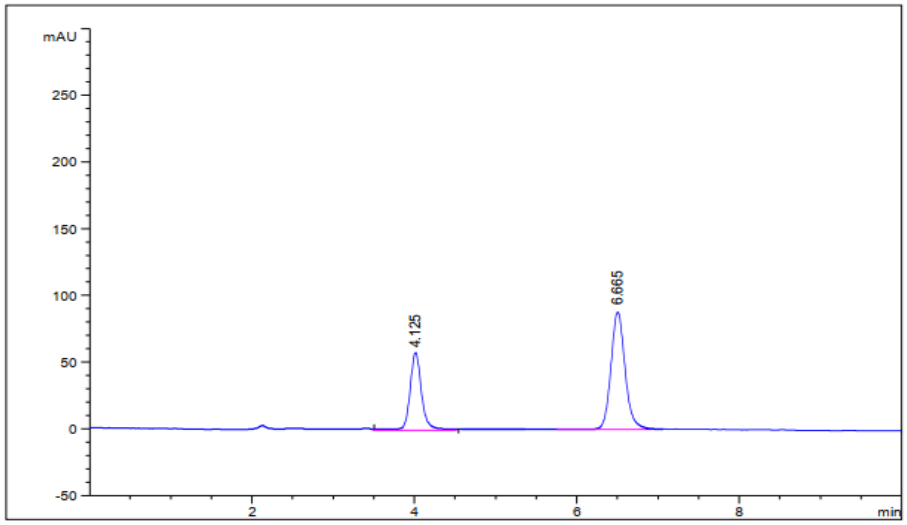

Figure 1: Chromatogram of Both the drug

Table 1: Linearity and Regression Parameters of DFP and MXF

Drug	Concentration Range (µg/ml)	Correlation Coefficient (r^2)	Slope (m)	Intercept (c)
DFP	1 – 5	0.999	1509.9	16.503
MXF	5 – 25	0.999	902.74	88.603

Table 2: System Suitability Parameters of DFP and MXF

Drug	Retention Time (RT) (min, Mean ± SD)	AUC (Mean ± SD)	Theoretical Plates (Mean ± SD)	Tailing Factor (Mean ± SD)
DFP	4.1167 ± 0.0043	7571.037 ±	2869.833 ± 22.887	1.182 ± 0.025

		15.507		
MXF	5.6665 ± 0.0048	9151.976 ± 9.671	2570.500 ± 14.580	1.147 ± 0.022

Table 3: Recovery (Accuracy) Study for DFP and MXF

Drug	Recovery Level	Mean % Recovery	SD	%RSD
DFP	80%	98.82	0.4337	0.4389
	100%	97.94	0.4194	0.4282
	120%	99.04	0.4520	0.4570
MXF	80%	98.23	0.800	0.814
	100%	98.20	1.047	1.066
	120%	98.41	0.798	0.811

Table 4: Precision Studies for DFP and MXF

Drug	Parameter	Mean % Assay	SD	%RSD
DFP	Repeatability	98.72	0.101	0.102
	Day-to-day precision	99.06	0.065	0.066
	Analyst-to-analyst precision	99.53	0.045	0.045
	Robustness	99.17	0.071	0.072
MXF	Repeatability	97.67	0.036	0.037
	Day-to-day precision	98.42	0.035	0.035
	Analyst-to-analyst precision	98.42	0.035	0.035
	Robustness	97.54	0.056	0.058

Table 5: LOD and LOQ of DFP and MXF

Name	LOD (µg/ml)	LOQ (µg/ml)
DFP	0.15	0.45
MXF	0.10	0.35

Table 6: Result of assay of tablet formulation

	DFP*	MXF*

Label Claim (mg)	**0.05%**	**0.5%**
% Found (mg)	0.048	0.49
% Assay	96.00	98.00
% RSD	0.048	0.49

Table 7: Results of Forced degradation studies of DFP

Stress conditions	**Drug recovered (%)**	**Drug decomposed (%)**
Standard drug	99.95	0
Acidic hydrolysis	89.98	9.97
Alkaline hydrolysis	93.32	6.63
Oxidative degradation	85.65	14.3
Photolytic degradation	94.45	5.5

Table 8: Results of Forced degradation studies of MXF

Stress conditions	**Drug recovered (%)**	**Drug decomposed (%)**
Standard drug	99.65	0
Acidic hydrolysis	91.15	8.5
Alkaline hydrolysis	94.78	4.87
Oxidative degradation	92.36	7.29
Photolytic degradation	96.45	3.2

Conclusion

A simple, accurate, precise, and stability-indicating RP-HPLC method was successfully developed and validated for the simultaneous estimation of Difluprednate and Moxifloxacin in ophthalmic formulations. The method complied with ICH guidelines, demonstrated excellent linearity, accuracy, precision, robustness, and sensitivity, and effectively separated degradation products from the analytes. Therefore, the proposed method is suitable for routine quality control and stability analysis of Difluprednate and Moxifloxacin.

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