RESEARCH ARTICLE

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF MOXIFLOXACIN AND DIFLUPREDNATE IN THEIR COMBINED DOSAGE FORM

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ABSTRACT

Moxifloxacin is synthetic fluoroquinolone antibiotic agent and Difluprednate is a topical corticosteroid indicated for the treatment of infammation and pain associated with ocular surgery. This combination of drugs will be used to treat optical infections. In RP-HPLC method, a mobile phase of Phosphate Buffer (pH 3.5) : Methanol (35:65 v/v) was used to resolve Moxifloxacin and Difluprednate from a mixture. The linearity range obtained for the HPLC method were 25 - 75 µg/ml and 2.5 – 7.5 µg/ml with corresponding correlation coefficient of 0.998 and 0.997, for Moxifloxacin and Difluprednate respectively. Flow rate was set to 1 ml/min and detection was carried out at 244 nm. The method was found to be rapid, accurate and precise. This method was validated according to ICH guidlines.

**Keywords:** RP-HPLC, Simultaneous Estimation, Moxifloxacin & Difluprednate

1. INTRODUCTION

The bactericidal action of moxifloxacin results from inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV. DNA gyrase is an essential enzyme that is involved in the replication, transcription and repair of bacterial DNA. Topoisomerase IV is an enzyme known to play a key role in the partitioning of the chromosomal DNA during bacterial cell division. Difluprednate is a topical corticosteroid indicated for the treatment of infammation and pain associated with ocular surgery. This combination of drugs will be used to treat optical infections.

The literature is enriched with several methods for determination of MOX and DIFLU in pharmaceutical dosage forms either as a single drug or in combination with some other drugs. The most extensively used technique for estimation of MOX are by UV[3-6], HPLC[7-14], and UPLC[15] methods and most extensively used technique for estimation of DIFLU is by HPLC[16]. The aim of study is development and Validation of Analytical HPLC method for Simultaneous Estimation of Moxifloxacin and Difluprednate in their combimed Dosage form.

The present study was designed to develop a simple, precise, and rapid analytical RP-HPLC procedure, which can be used for the analysis of assay method for simultaneous estimation of Moxifloxacin and Difluprednate as there was only individual methods reported for both drugs. The combination of these two drugs is not official in any pharmacopoeia; hence no official method is available for the simultaneous estimation of these two drugs in their combined dosage forms. Literature survey of Moxifloxacin and Difluprednate revealed several methods for detecting these drugs individually but there is no method for their simultaneous estimation using RP-HPLC.
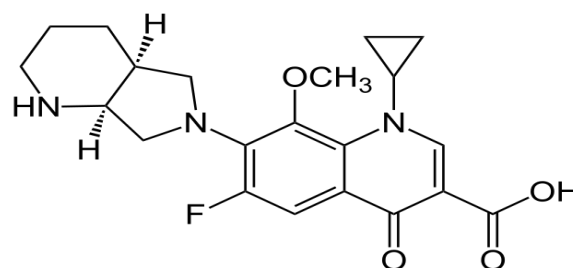
FIGURE 1: Chemical structure of Moxifloxacin
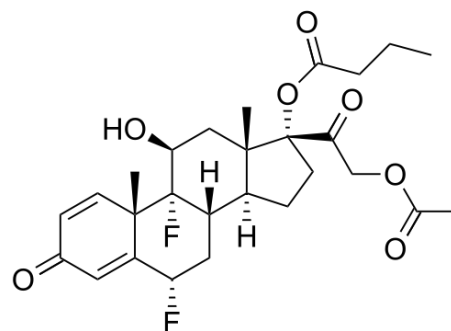
FIGURE 2: Chemical structure of Difluprednate

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2. MATERIAL AND METHODS

2.1 Apparatus and Instrument

The analysis was carried out on a HPLC system (Shimadzu LC-20- AT) equipped with UV detector. Other apparatus and instruments used were a micro analytical balance (Shimadzu), Ultrasonic Cleaner (EIE Instruments Pvt. Ltd. Ahmedabad), Nylon Membrane Filters (0.22mcm, 47 mm D). All instruments and glass wares were calibrated.

2.2 Reagents and Materials

Moxifloxacin and Difluprednate were obtained as gratis sample from Accurate Pharmaceuticals, Godhra. Methanol HPLC Grade, Water HPLC Grade, Phosphate Buffer , O-Phosphoric Acid were used which were obtained from Samir Tech-Chem Pvt. Ltd. A stock-standard solution of MOX and DIFLU was prepared by dissolving accurately weighed amount of pure drug in mobie phase.

2.3 Mobile Phase:
Phosphate Buffer (pH 3.5) : Methanol(35:65 v/v). The mobile phase was filtered through Millipore filter paper type HV (0.45 μm) and degassed by sonication.

2.4 Chromatographic conditions

Chromatographic analysis was carried out on an an inertsil C-18 column, (5 μm, 250mm x 4.6mm i.d) LC-20 AT. The mobile phase consisted of Phosphate Buffer(pH 3.5) : Methanol(35:65 v/v). The mobile phase was filtered through Millipore filter paper type HV (0.45 μm) and degassed by sonication, was pumped at 1.0 ml/min flow rate. The column was thermostated at room temperature. Under these conditions the runtime was 10 min.

2.4.1 Preparation of standard stock solution of MOX (500 μg/ml) and DIFLU (50μg/ml)

A 50 mg of standard MOX and 5 mg of standard DIFLU was weighed and transferred to a 100 ml volumetric flask each and dissolved in 25 ml mobile phase. The flask was shaken and volume was made up to the mark with mobile phase to give a solution containing 500 μg/ml MOX and 50 μg/ml of DIFLU.

2.4.2 Preparation of combined working standard solution containing MOX and DIFLU in ratio of 10:1

Accurately weighed 50 mg MOX and 5 mg of DIFLU were transferred to 100 ml volumetric flask, dissolved in sufficient amount of mobile phase and diluted up to mark with mobile phase to get concentration of 500 μg/ml MOX and 50 μg/ml DIFLU. This solution was diluted

further to get the concentrations in range of 25, 37.5, 50, 62.5, 75 μg/ml Moxifloxacin and 2.5, 3.75, 5.0, 6.25, 7.5 μg/ml Difluprednate.

2.5 Method Validation

2.5.1 Precision

Repeatability: Precision of the method was studied by making repeated injections of the mixture of drugs on the same day for intraday precision.The %RSD after six determinations was determined at 50 μg/ml for MOX and 5 μg/ml for DIFLU.

Intraday and Interday Precision: Intraday and Interday precision for method were measured in term of %RSD. The experiment was repeated three times in a day for intraday and on three different days for interday precision by taking small, middle and higher concentration of MOX(25, 50, 75 μg/ml) and DIFLU(2.5, 5.0, 7.5 μg/ml).

2.5.2 Linearity:
The linearity of measurement was evaluated by analyzing standard solutions of MOX and DIFLU in the range of 25–75 μg/ml and 2.5-7.5 μg/ml for both drugs respectively and calibration plot was constructed.

2.5.3 Limit of Detection (LOD) and Limit of Quantitation (LOQ):

LOD and LOQ of MOX and DIFLU were determined by calibration curve method. Solutions of Moxifloxacin and Difluprednate were prepared in the range of 25–75 μg/ml and 2.5-7.5 μg/ml for both drugs respectively and injected in triplicate.

2.5.4 Accuracy:
Accuracy of the method was calculated by recovery studies at three levels by standard addition method, which is, spiking about 80%, 100%, 120% of MOX and DIFLU to the standard solutions containing 25 μg/ml of MOX and 2.5 μg/ml of DIFLU.

2.5.5 Robustness:
Influence of small changes in chromatographic conditions such as change in flow rate, that is, ±0.2 ml/min, mobile phase composition ±2 ml and pH ±0.2 was studied to determine the robustness of the method for the development of RP-HPLC method for the simultaneous estimation of MOX and DIFLU and their %RSD was determined.

2.5.6 System Suitability:
The stock solution containing 50 μg/ml of MOX and 5 μg/ml of DIFLU was injected and repeated five times and the chromatograms were recorded.The resolution, number of theoretical plates, and peak asymmetry were calculated to determine whether the result complies with the recommended limit.
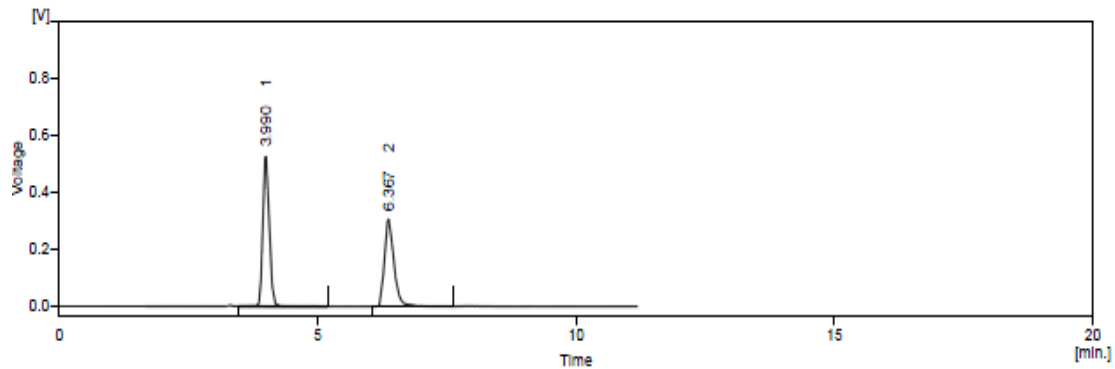
3. RESULTS AND DISCUSSION

FIGURE 3: Chromatogram of MOX and DIFLU respectively

3.1 Optimization of Chromatographic conditions

To optimize the chromatographic conditions for separation of Moxifloxacin and Difluprednate, mobile phase composition, the effect of temperature and wavelength of detection investigated.

During the method development work, enable C18 column (25 cm× 4.6 mm i.d.) with particle size of 5 μm was used and gave the suitable resolution. The mobile phase composition was prepared with appropriate proportion of phosphate buffer (Ph 3.5) : Methanol (35:65 v/v).

It was shown that the most efficient resolution and peak symmetry for Moxifloxacin and Difluprednate with a mobile phase composed of phosphate buffer (Ph 3.5) :

Methanol (35:65 v/v) and a flow rate of 1 ml/min. The retention time for Moxifloxacin and Difluprednate were found to be 3.99 and 6.637 min respectively.

The wavelength selected was from the overlay of Moxifloxacin and Difluprednate. Both the drugs showed typical peak nature and peaks were symmetrical at 244 nm. Hence the wavelength has been selected as the detection wavelength.

3.2 Validation

3.2.1 Linearity and Range

The linearity of measurement was evaluated by analyzing standard solutions of MOX and DIFLU in the range of 25–75 μg/ml and 2.5-7.5 μg/ml respectively for both drugs and calibration plot was constructed.

TABLE 1: Statistical Parameter for Moxifloxain and Difluprednate

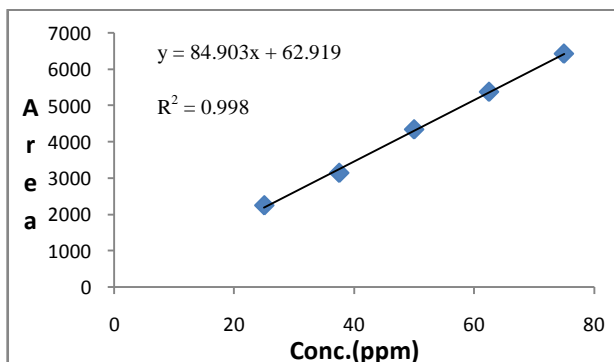| Statistical Parameter	Moxifloxacin	Difluprednate
Average peak area* ± SD	2245.07 ± 1.54	1882.85 ± 1.73
	3141.42 ± 1.56	2602.19 ± 1.66
	4342.16 ± 1.58	3641.42 ± 1.65
	5378.89 ± 1.14	4510.82 ± 1.58
	6432.78 ± 1.80	5394.70 ± 1.51
Concentration Range (μg/ml)	25-75	2.5-7.5
Straight line equation	y = 84.903x + 62.919	y = 714.59x + 33.467
Correlation coefficient (R^2)	0.998	0.997
LOD (μg/ml)	2.83	0.35
LOQ (μg/ml)	8.57	1.07

n=5

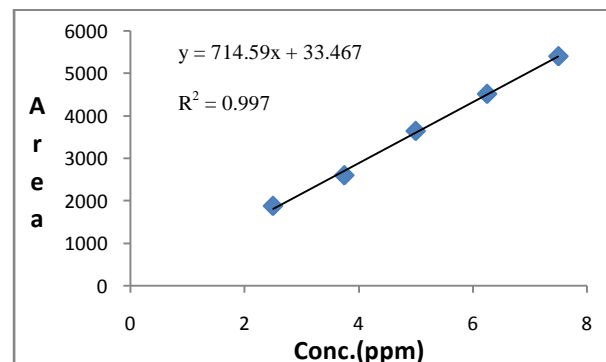**FIGURE 4: Calibration curve of Moxifloxacin**

FIGURE 5: Calibration curve of Difluprednate

3.2.2 Precision

Precision of the method was studied by making repeated injections of the mixture of drugs. The Relative Standard

Deviation (%RSD) after six determinations was 0.67% at 50 µg/ml for MOX and 1.23% at 5 µg/ml for DIFLU (see Table 2).

TABLE 2: Precision data for MOX and DIFLU

Precision	Concentration found* (µg/ml)		%RSD	
	MOX	**DIFLU**	**MOX**	**DIFLU**
Repeatibility	50.07	4.93	0.57	0.86
Intraday Precision	25.01	2.51	0.69	1.12
	50	5.01	1.16	1.37
	75.99	7.59	0.49	1.24
Interday Precision	25	2.51	0.79	1.55
	49.99	5.01	0.69	1.70
	75.01	7.5	0.30	0.81

n=6

3.2.3 LOD and LOQ

LOD and LOQ of MOX and DIFLU were determined by calibration curve method. Solutions of MOX and DIFLU were prepared in the range of 25–75 µg/ml and 2.5-7.5 µg/ml respectively and injected in triplicate (see Table 1).

3.2.4 Accuracy

Accuracy of the method was calculated by recovery studies at three levels by standard addition method. The mean percentage recoveries obtained for MOX and DIFLU were 99.59% and 99.72%, respectively (see Table 3).

TABLE 3: Recovery data for MOX and DIFLU

Diflumox Eye Drops	Conc. in eye drops (µg/ml)	Conc. added (µg/ml)	Total conc. found (µg/ml)	Amount recovered (µg/ml)	Mean Recovery* ± SD (%)
Moxifloxacin	25	20	44.99	19.99	99.61 ± 0.79
	25	25	49.90	24.90	99.57 ± 0.53
	25	30	54.90	29.90	99.59 ± 0.41
Difluprednate	2.5	2	4.47	1.97	99.92 ± 0.64
	2.5	2.5	4.99	2.49	99.63 ± 0.65
	2.5	3	5.49	2.99	99.63 ± 0.49

n=3

3.2.5 Robustness

The method for the development of RP-HPLC method for the simultaneous estimation of MOX and DIFLU was

found to be robust as the % RSD was found to be less than 2 (see Table 4)

TABLE 4: Robustness data for MOX and DIFLU

Parameters	Variation	% RSD	
		MOX	**DIFLU**
Flow rate	1.2	0.80	1.64
	(1 ml/min)	0.56	0.85
	0.8	0.88	1.59
Mobile phase	(37:63)	1.22	0.66
	Phosphate buffer pH 3.5 : Methanol (35:65)	0.56	0.85
	(33:67)	0.83	1.77
pH (3)	(3.2)	1.21	1.74
	pH (3)	0.56	0.85
	(2.8)	0.95	0.38

3.2.6 System Suitability

The resolution, number of theoretical plates, and peak asymmetry were calculated for the standard solutions.

The stock solution containing 50 µg/ml of MOX and 5 µg/ml of DIFLU was injected and repeated five times and the chromatograms were recorded. The resolution, number of theoretical plates, and peak asymmetry were

calculated to determine whether the result complies with

the recommended limit (see Table 5)

TABLE 5: System Suitability Parameters

Parameters	Drugs	
	MOXIFLOXACIN	**DIFLUPREDNATE**
Retention Time (min)	3.99	6.367
Resolution (Rs)	8.391	
Tailing Factor (t)	1.34	1.54
No. of theoretical plates	4500	6008

TABLE 6: Summary of Validation Parameter:

Sr. No.	Parameters	Results	
		MOXIFLOXACIN	**DIFLUPREDNATE**
1.	Linearity Range (n=5) (μg/ml)	25-75	2.5-7.5
2.	Regression equation	y = 84.903x + 62.919	y = 714.59x + 33.467
3.	Correlation coefficient (R^2)	0.998	0.997
4.	Limit of detection (n=5) (μg/ml)	2.83	0.35
5.	Limit of quantification (n=5) (μg/ml)	8.57	1.07
6.	Precision		
	Repeatability (%RSD) (n=6)	0.57 %	0.86 %
	Intraday (%RSD)(n=3)	0.78 %	1.24 %
	Interday (%RSD)(n=3)	0.59 %	1.35 %
7.	Robustness (%RSD) (n=3)	0.985 %	1.303 %
8.	Accuracy (Mean ± SD) (%, n=3)	99.59 ± 0.57	99.72 ± 0.59

3.3 Procedure for the analysis of Eye Drops:

Sample: Moxifloxacin and Difluprednate

Brand name: DIFLUMOX (Moxifloxacin 5%, Difluprednate 0.5%)

Manufacturer: Ajanta Pharma Ltd, Mumbai, Maharashtra, India.

3.3.1 Preperation of sample solution:

DIFLUMOX eye drops containing 5 % of MOX and 0.5 % of DIFLU is available in local market which is marketed by Ajanta Pharma(Mumbai). It contains 50 mg

of MOX and 5 mg of DIFLU per ml. For preparation of stock solution, 1 ml from the suspension was taken in 10 ml volumetric flask and diluted up to 10 ml with mobile phase. Then it was filtered through whatman filter paper (0.45μ). It was concentration of 5000 μg/ml of MOX and 500 μg/ml of DIFLU. From that 1 ml of aliquots was taken and diluted up to 10 ml for getting concentration of 500 μg/ml of MOX and 50 μg/ml of DIFLU. From this stock solution, working standard solution of 50 μg/ml of MOX and 5 μg/ml of DIFLU was prepared by taking 1 ml and diluted it up to 10 ml with mobile phase. This solution was used for the estimation of MOX and DIFLU in their combined dosage form.

TABLE 7: Analysis of market formulation

Drug	Label claim (%)	Conc. taken for % Assay (μg/ml)	Average Peak Area*	Conc. found from Mixture as per labe1 claim (%)	Assay* ± SD (%)
Moxifloxacin	5	50	4453.85	4.93%	98.60 ± 0.74
Difluprednate	0.5	5	3825.01	0.504%	100.98 ± 1.20

n=3

CONCLUSION

The proposed RP-HPLC method was used for the simultaneous estimation of Moxifloxacin and Difluprednate was found to be sensitive, accurate, precise, simple, and rapid. Hence the present RP-HPLC method may be used for routine analysis of the

raw materials as well as combined dosage formulations containing Moxifloxacin and Difluprednate.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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