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Simultaneous estimation of moxifloxacin hydrochloride and difluprednate in ophthalmic formulation by three novel spectrophotometric methods

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ABSTRACT

Three simple, rapid and accurate spectrophotometric methods were developed for the simultaneous determination of moxifloxacin hydrochloride and difluprednate in ophthalmic eye drop formulation using methanol as solvent. Method 1 is the simultaneous estimation method and both the drugs exhibit good linearity over the concentration range of 4 to 12 µg/ml for moxifloxacin and 0.4 to 1.2 µg/ml for difluprednate at λ_{max} of 291.0 and 237.0 nm, with regression coefficient 0.9993 and 0.9953 respectively. Method 2 is the second order derivative method, where moxifloxacin and difluprednate showed zero cross over point at 240.0 and 252.6 nm respectively. Beer's Lambert's law was obeyed over the concentration range of 4 to 12 µg/ml for moxifloxacin and 1 to 12 µg/ml for difluprednate with regression coefficient 0.9991 and 0.9945. Method 3 is the ratio second derivative method, and at 275.20 and 249.40 nm, linear concentration range for moxifloxacin and difluprednate was 4 to 10 µg/ml and 4 to 20 µg/ml with regression coefficient 0.9976 and 0.9952 respectively. The methods were validated according to ICH guidelines for evaluation of accuracy, precision, repeatability, reproducibility, sensitivity etc. The proposed procedures can be successfully applied for the determination of moxifloxacin and difluprednate in eye drops, for routine quality control analysis.

Key words: Moxifloxacin hydrochloride, Difluprednate, Simultaneous estimation, Second derivative method, ratio second derivative ratio method, validation

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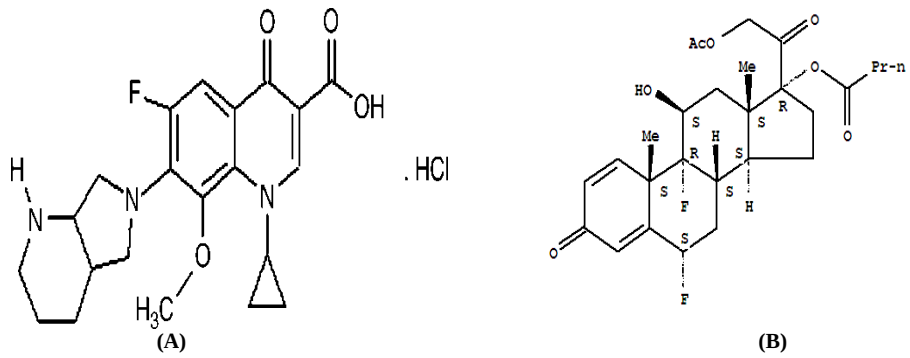
INTRODUCTION

Moxifloxacin hydrochloride (MOXI) is a fourth generation 8-methoxy fluoroquinolone derivative [1-cyclopropyl-6-fluoro-1, 4-dihydro-8-methoxy-7-{(4aS, 7aS–octa-hydro-6H-pyrrolol (3,4b) pyridin6-yl)}-4-oxo-3-quinoline carboxylic acid, monohydrochloride] (Figure 1a). Moxifloxacin is a broad-spectrum antibiotic active against both gram-positive and gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II Topoisomerase, and Topoisomerase IV, which is an enzyme necessary to separate replicated DNA, thereby inhibiting cell division [1-4]. Difluprednate (DIFLU), a synthetic glucocorticoid, chemically is DFBA, 6a, 9-difluoro-11b, 17, 21-trihydroxypregna-1, 4-diene-3, 20-dione 17-butyrate 21-acetate (Figure 1b). It is also used for the treatment of inflammation and pain associated with ocular surgery.



(A) **(B)**

Figure 1: Chemical structure of (A) Moxifloxacin hydrochloride (B) Difluprednate

Literature reviewed reports several analytical methods like spectrophotometry, HPLC, HPTLC [5-11], for the determination of individual drugs, MOXI and DIFLU but none of the analytical method is available for determination of combination of both the drugs in eye drop formulation. In context to this, this research paper describes UV spectrophotometric methods that find wide application for the determination of MOXI and DIFLU in ophthalmic formulation. The spectrophotometric analysis of drugs rarely involves measurement of absorbance of sample containing only one absorbing component. Simultaneous quantitative analysis of multicomponent mixture is difficult to perform by classical spectrophotometric method due to overlapping spectra [12]. However, spectrophotometric technique combined with wavelength transformation has brought a new, fast and easy to apply methodology for the determination of analytes in complex samples without prior separation [13-16]. The enhanced resolution and bandwidth discrimination increases with increasing derivative order. The research paper herewith describes the development of three UV spectrophotometric methods, simple UV method for binary mixture (method 1), second order derivative method (method 2) and ratio second order derivative method (method 3). All the three proposed methods are economically viable, more rapid and novel methods that do not require any prior separation procedure. All developed UV methods are validated as per ICH guidelines (Q2R1), meeting required criteria for specificity, accuracy, precision and are suitable for routine quality control analysis of MOXI and DIFLU in ophthalmic dosage form.

MATERIALS AND METHODS

Materials and chemicals

MOXI and DIFLU were obtained as a gift sample from Nivika Chemo Pharma, Ankleshwar and Ajanta Pharma Limited, Mumbai respectively. Formulation Diflumox eye drops containing 5 mg of MOXI and 0.5 mg of DIFLU per each ml (Ajanta Pharma Limited, Mumbai), was procured from local market. All solvents and chemicals used were of analytical grade, purchased from Merck Specialities Pvt. Ltd., India.

Instrumentation

A double beam UV-Visible spectrophotometer (Shimadzu, Japan) model UV-1800 with a quartz cell of 1 cm path length and fixed slit width (2nm), UV probe software (Shimadzu version 2.34), electronic analytical balance (AUX-220D, Shimadzu) and Ultrasonic cleaner (USC 100) Toshniwal process instrument Pvt. Ltd. were used in the study.

Spectrophotometric conditions

For proposed methods 1, 2 and 3; spectrum mode with medium scan speed was adjusted in double beam UV spectrophotometer. The wavelength range of 400-200 nm and absorbance scale of 0.00A–4.00A was selected. The initial baseline correction was done by using methanol. Second order derivative mode was selected for method-2 and method-3.

Preparation of stock and working standard solution for MOXI and DIFLU

Method-1: Binary stock solution of MOXI and DIFLU was prepared by an accurately weighed quantity of MOXI (25 mg) and DIFLU (2.5 mg) in to 25 ml volumetric flask, dissolved and diluted up to mark with methanol to obtain a final concentration of 1000 µg/ml MOXI and 100 µg/ml DIFLU. Further dilution was performed with methanol, to prepare final working standard solution containing 10 µg/ml and 1 µg/ml of MOXI and DIFLU respectively.

Method-2 and Method-3: Stock solution of MOXI and DIFLU was prepared by an accurately weighed quantity of MOXI (25 mg) and DIFLU (25 mg), by dissolving in small quantity of methanol separately and further sonicated for 15 min. The volume was finally made up to 25 ml in volumetric flask to give a final concentration of 1000 µg/ml

MOXI and DIFLU. From stock solutions, appropriate dilution was made with methanol to obtain a final concentration of MOXI (10 µg/ml) and DIFLU (10 µg/ml).

Spectrophotometric methods

Method-1:

From working standard solution, appropriate aliquots were diluted up to 10 ml in volumetric flask with methanol to prepare final concentration in the range of 4-12 µg/ml for MOXI and 0.4-1.2 µg/ml for DIFLU. Absorbance was measured at the selected wavelength of maximum absorption for MOXI (291.0 nm) and DIFLU (237.60 nm), in 1cm cell against methanol as blank.

Method-2:

Appropriate aliquots from the working standard solution were transferred and diluted up to 10 ml in volumetric flask with methanol to prepare final concentration range of 4 to 12 µg/ml for MOXI and 1 to 12 µg/ml for DIFLU. Zero order spectra were recorded for all calibration standard solution for both MOXI and DIFLU and second order derivative spectra were obtained by transformation using $\Delta\lambda = 8$ and scaling factor 10. Absorbance for estimation of DIFLU and MOXI was measured at the selected wavelength of zero cross over point, 240.0 nm and 252.6 nm for MOXI and DIFLU respectively.

Method-3:

Different aliquots from the standard solution were transferred into 10 ml volumetric flask and diluted with methanol to prepare final concentration in the range of 4-10 µg/ml for MOXI and 4-20 µg/ml for DIFLU. Zero order spectra were recorded for calibration standard solution of MOXI and DIFLU and were stored. Further, zero order absorption spectra of MOXI were divided by the spectrum of the standard solution of DIFLU (10 µg/ml). Similarly, zero order absorption spectra of DIFLU were divided by the standard spectrum of MOXI (10 µg/ml). All spectra were stored in the IBM-PC. The first derivative of the ratio spectra of both MOXI and DIFLU were recorded by transformation using $\Delta\lambda = 8$ and scaling factor 10. Absorbance for MOXI and DIFLU was obtained by measuring the amplitude at the selected wavelength of 275.2 nm and 249.4 nm respectively.

Validation of method

Proposed methods were validated in accordance with ICH guidelines Q2 (R1) for evaluation of various parameters; linearity, precision, accuracy, limit of detection, limit of quantification, specificity and robustness [17].

Linearity and Range:

Linear relationship between absorbance and concentration of MOXI and DIFLU were evaluated over the concentration range for method-1, method-2 and method-3 by making five replicate measurements. Calibration plots were constructed by plotting the absorbance versus the concentration and treated using the method of ordinary least squares regression analysis. Moreover, linearity was also validated by applying "Bartlett's test" for homoscedasticity of variance.

LOD and LOQ:

As per ICH guideline, limit of detection and quantification of the developed method were calculated from the standard deviation of the response and slope of the calibration curve of each drug using the formula,

$$\text{Limit of detection} = 3.3 \times \sigma / S$$
$$\text{Limit of quantification} = 10 \times \sigma / S$$

Where, "σ" is standard deviation of response
"S" is Slope of calibration curve

Precision:

Precision of the developed methods were evaluated by performing repeatability on the same day and intermediate precision studies on different days in three replicates. Repeatability and intermediate precision was performed for MOXI at 4, 8, 12 µg/ml (method-1 and method-2), and 2, 6, 10 µg/ml (method-3), and for DIFLU at 0.4, 0.8, 1.2 µg/ml (method-1), 2, 6, 8 µg/ml (method-2); 4, 14, 20 µg/ml (method-3) and absorbance measured was expressed in terms of percent relative standard deviation (% RSD).

Accuracy:

Accuracy of method was ascertained by performing recovery study by standard addition method at three concentration levels (50%, 100% and 150%) in triplicate. For MOXI, three different concentrations of MOXI standard (2, 4 and 6 µg/ml) were spiked to the formulation (4 µg/ml) for method-1, 2 and 3. Similarly, recovery

studies for DIFLU were carried out by spiking three different concentrations of DIFLU standard (0.2, 0.4 and 0.6 µg/ml) to the formulation (0.4 µg/ml) for method-1, (0.5, 1.0 and 1.5 µg/ml) to the formulation (1 µg/ml) for method-2 and (4, 8 and 12 µg/ml) to the formulation (8 µg/ml) for method-3.

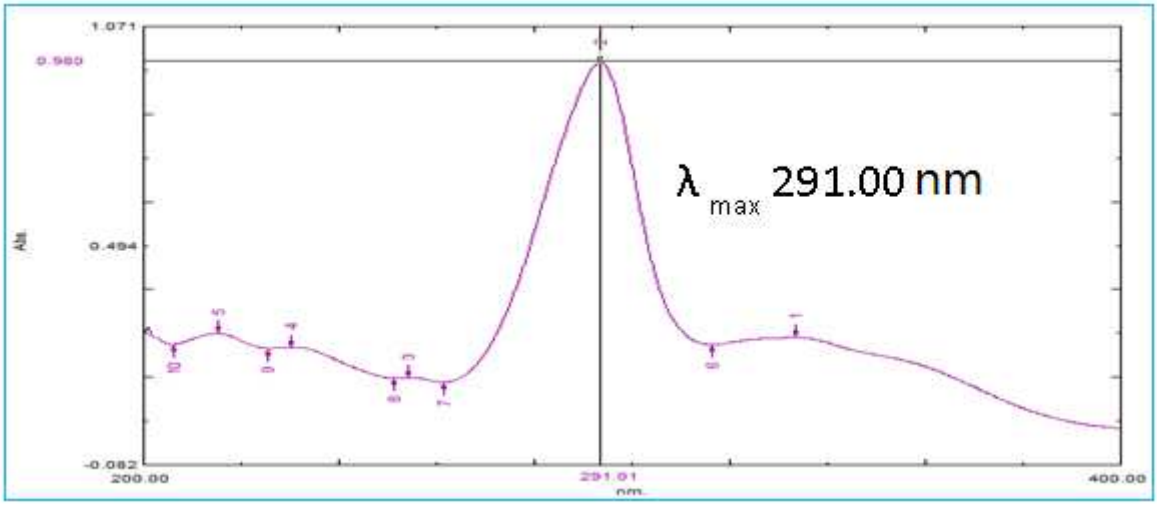
Applicability of the proposed methods for analysis of ophthalmic dosage formulation:
An accurately measured volume of eye drop formulation equivalent to 25 mg of MOXI and 5 mg of DIFLU was transferred into 25 ml volumetric flask, followed by addition of 20 ml methanol. The mixture was sonicated for 15 min and then filtered through Whatman filter paper no. 42, wetted with methanol. The volume was finally made up to the mark with methanol. The solution was further diluted with methanol to obtain the concentration of MOXI (10 µg/ml) and DIFLU (1 µg/ml) and the method described above for method-1 and 2 was then applied for determination of absorbance and triplicate analysis was performed by following the same procedure. While for method 3, the solution was further diluted with methanol to obtain required concentration of MOXI (12 µg/ml) and DIFLU (1.2 µg/ml) and the method described above was then applied for determination of absorbance in triplicate.

Statistical analysis:
Statistical parameters like SD, %RSD were computed by using MS Excel. Bartlett's test was applied on the data of linearity for evaluation of homoscedasticity of variance [18].

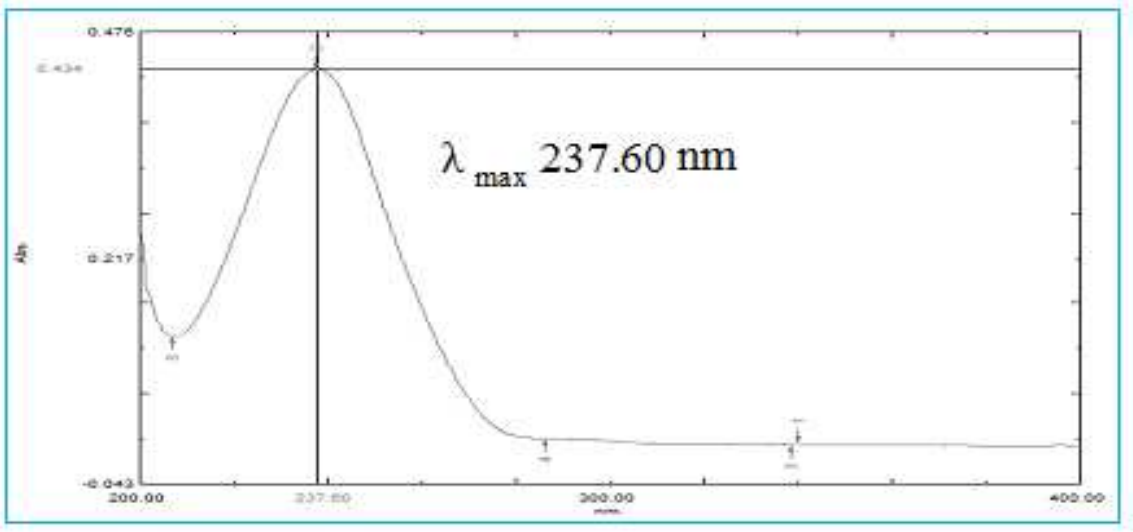
RESULTS AND DISCUSSION

The proposed methods, simple UV method, second order derivative spectrophotometric method and ratio second order derivative method were developed and applied for the simultaneous determination of MOXI and DIFLU in the binary mixture without prior separation steps.

Method development and optimization
The zero-order absorption spectra of MOXI and DIFLU showed wavelength of maximum absorption for MOXI and DIFLU at 291.0 nm and 237.6 nm respectively that was selected for simultaneous estimation of both the drugs in method 1 (Figure 2A and B). However, zero order absorption spectra did not show complete resolution, hence derivative and ratio derivative spectrophotometric method were explored. The second order derivative spectra showed good resolution with zero cross over point for MOXI and DIFLU at 240.0 and 252.6 nm for simultaneous determination instead of first order derivative (Figure 3).



(A)



(B)

Figure 2: Zero order absorption spectra showing wavelength of maximum absorption for (A) MOXI (λ_{max} 291.0 nm), (B) DIFLU (λ_{max} 237.6 nm)

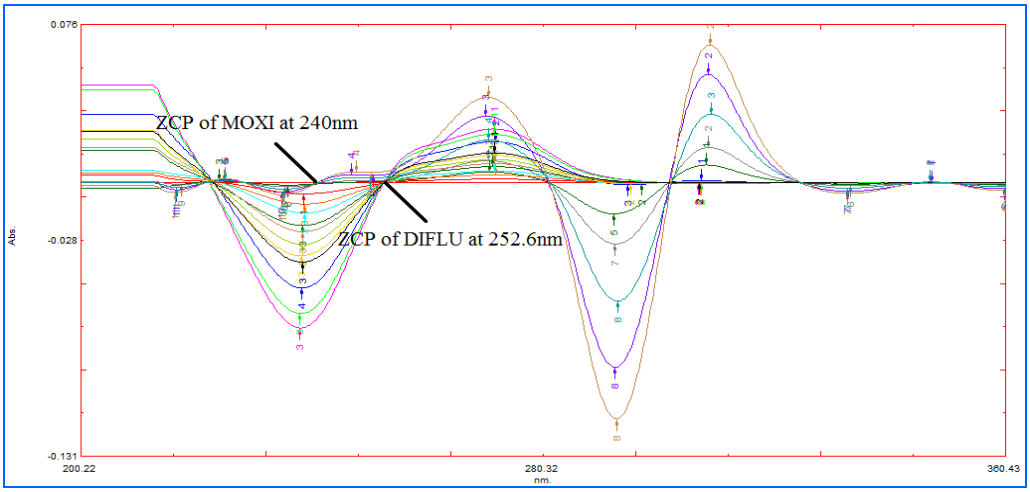
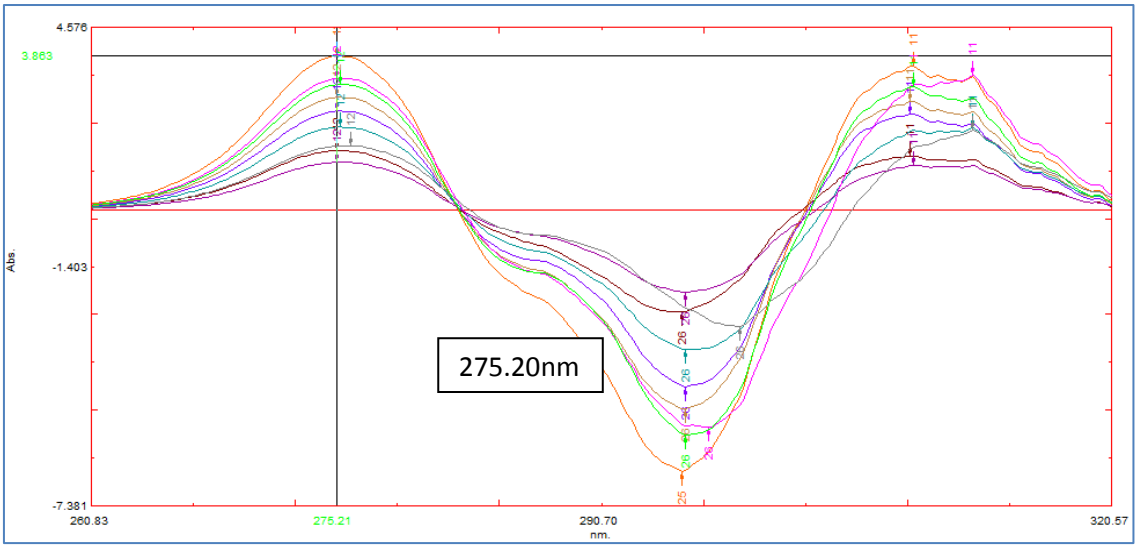
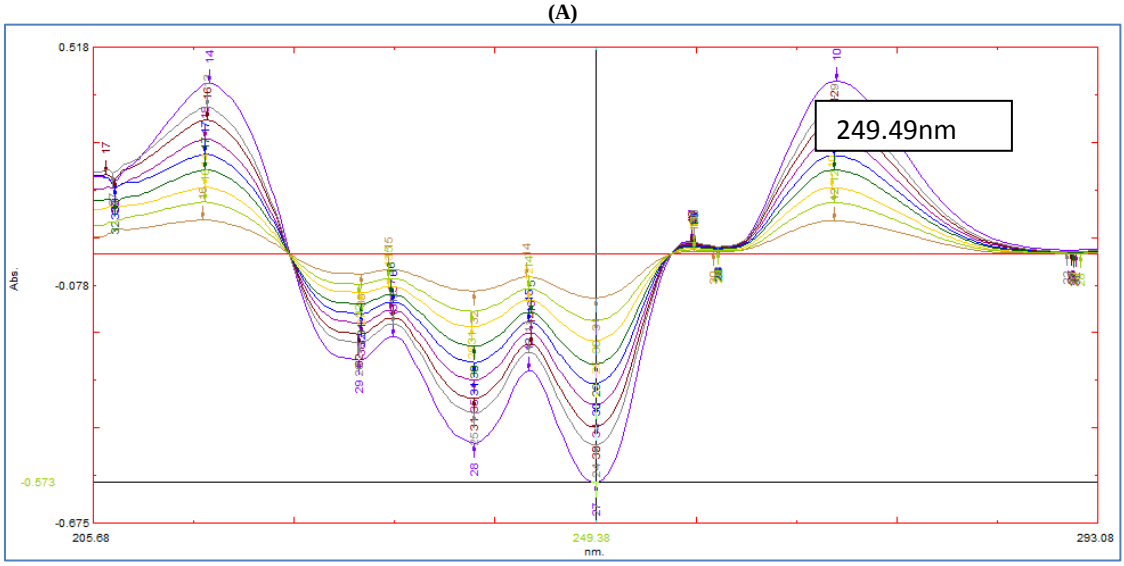


Figure 3: Second order derivative spectra of MOXI and DIFLU standard showing ZCP at 240.0 nm and 252.6 nm respectively

The third method ratio derivative method has various advantages of easy measurements on separate peaks, higher values of analytical signals, and no need to work at zero cross over point. The effect of divisor concentration on the analytical parameters such as slope, intercept and correlation coefficient was also tested. The chosen divisor concentration gave good results for the slope, intercept and correlation coefficient of calibration graphs as well as for selectivity. Ratio spectra of different concentration of MOXI standards (spectra divided by standard spectrum of a 10 µg/ml solution of DIFLU) and their second order derivative, shows the height of the maximum at 275.2 nm for MOXI for its estimation (Figure 4A). Similarly, ratio spectra of different DIFLU standards (spectra divided by standard spectrum of a 10 µg/ml MOXI solution), and corresponding second order derivative spectra shows maximum amplitude at 249.40 nm for DIFLU for its quantification (Figure 4b).



(A)



(B)

Figure 4: Ratio second order derivative spectra showing wavelength of maximum absorption for (A) MOXI and (B) DIFLU at 275.2 nm and 249.4 nm respectively

Validation of proposed methods

The proposed methods have been validated for repeatability, reproducibility, intermediate precision, accuracy, specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ). The calibration curves were constructed for the proposed methods according to their respective concentration ranges and were found to be linear over the concentration range for MOXI and DIFLU with acceptable regression coefficient as shown in Table 1 for three proposed methods. Further homoscedasticity of variance for response, absorbance with respect to concentration range for all the three methods for MOXI and DIFLU was also validated by Bartlett's test (Table 1). The results showed that the calculated $\chi 2$ value is less than the critical value at 95% confidence interval, $\chi 2_{(0.05, 5)} = 9.488$; thus indicating that the variance of response is homogeneous.

Repeatability and intermediate precision studies showed % RSD < 2, thus demonstrating good repeatability and reproducibility of the proposed methods (Table 1). Specificity of the proposed methods is evident from the spectra shown in Figure 3 and 4a-b. Recovery study by spiking the standard at 3 concentration levels, 50, 100 and 150 %, showed % RSD of less than 2% with acceptable percent recovery, indicating that the proposed method is accurate and can be applicable for routine analysis of formulation (Table 1). Both the drugs were stable over a period of 24 h at room temperature in methanol.

Table 1: Analytical parameters of MOXI and DIFLU for the proposed methods

Parameters	Method 1		Method 2		Method 3	
	MOXI	DIFLU	MOXI	DIFLU	MOXI	DIFLU
Wavelength (nm)	291.0	237.6	252.6	240.0	275.2	249.4
Range (µg/ml)	4-12	0.4-1.2	4 - 12	1 - 12	4-10	4-20
Correlation coefficient(r)	0.9993	0.9953	0.9991	0.9945	0.9976	0.9952
Slope ± SD[a](S_a)	0.1067 ± 0.0005	0.2757 ± 0.0015	0.00056± 0.000005	0.0026± 0.00003	0.3261 ± 0.0004	0.0277 ± 0.00004
Intercept ± SD[a](S_a)	0.0097 ± 0.0005	0.0054 ± 0.0012	0.00016 ± 0.00003	0.0015± 0.00011	0.1379 ± 0.0034	0.0277 ± 0.0004
Limit of detection (µg/ml)	0.088	0.014	0.194	0.139	0.035	0.051
Limit of quantification (µg/ml)	0.267	0.042	0.588	0.422	0.105	0.154
Bartlett's test[b] (χ^2)	0.0025	0.0019	0.0012	0.0082	0.00002	0.0003
Precision[c](% RSD)						
Repeatability	0.021-0.054	0.135-0.289	0.142-0.789	0.764-0.977	0.041-0.172	0.043-0.063
Intermediate precision	0.131-0.501	0.148-0.294	0.684-1.358	0.939-1.128	0.415-0.469	0.168-0.663
Accuracy[d]						
50%	101.00 ± 1.12	98.28 ± 0.44	99.28 ± 0.16	100.45 ± 1.43	101.52 ± 0.80	99.29 ± 0.23
100%	100.65 ± 0.13	100.22 ±0.53	99.81 ± 0.02	100.45 ± 1.43	100.22 ± 0.13	100.18 ± 0.34
150%	99.42 ± 0.16	98.85 ± 0.92	101.19 ± 0.18	98.64 ± 0.14	99.48 ± 0.12	101.93 ± 0.18

[a] Average of five determinations, [b] Calculated value less than tabulated value, 9.488 at 95% confidence interval, [c] Average of three determinations for each concentration, [d] Average of three determinations at each level

Analysis of ophthalmic dosage formulation

The marketed formulation, eye drops, Diflumox (label claim), when analyzed in triplicate using the developed methods, showed no interference of the excipients. The content of MOXI was in the range of 99.73-100.12% and for DIFLU in the range of 100.08-100.43%, which proves applicability of the developed methods in routine analysis of pharmaceutical eye drop formulation (Table 2).

Table 2: Percent amount of MOXI and DIFLU in ophthalmic formulation by proposed methods

Method	Drug	Label claim	% Mean of drug found [a]	% RSD
1	MOXI	5 mg/ml	100.12	0.539
	DIFLU	0.5 mg/ml	100.43	0.402
2	MOXI	5 mg/ml	99.94	0.557
	DIFLU	0.5 mg/ml	100.08	0.191
3	MOXI	5 mg/ml	99.73	0.341
	DIFLU	0.5 mg/ml	100.08	0.284

[a] average of three determinations

CONCLUSION

The methods developed are simple that does not require prior separation, rapid and direct as it estimates each drug independently of the other. The proposed methods are found to be precise, accurate and sensitive as revealed from % RSD less than 2, for simultaneous quantitative estimation of moxifloxacin hydrochloride and difluprednate in multicomponent mixture over the applied range. The three suggested methods were statistically compared using the analysis of variance (ANOVA) test. Statistical analysis using one way ANOVA at 95% confidence interval was performed. The p value showed that there is no significant difference between the proposed methods (0.6505 for MOXI and 0.3316 for DIFLU). F value calculated for MOXI (0.4623), and for DIFLU (1.3341), was found to be less than the tabulated F value (5.143). The test ascertains that the proposed methods are precise and accurate and comparable to one another. The results obtained indicate that the introduced methods can be classified amongst highly selective and sensitive procedures. These merits suggest the use of the proposed method in routine and quality control analysis without interference of commonly encountered dosage form additives.

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