Chromatographic Methods for Simultaneous Determination of Moxifloxacin Hydrochloride and Difluprednate in Ophthalmic Dosage Form

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Summary. Two simple, accurate, specific, and precise chromatographic methods, reversed phase high-performance liquid chromatography (RP-HPLC) and high-performance thin-layer chromatography (HPTLC), have been developed and validated for the determination of moxifloxacin hydrochloride and difluprednate in ophthalmic dosage form according to International Conference on Harmonization (ICH) guidelines. The separation of moxifloxacin hydrochloride and difluprednate in HPLC was performed on reverse phase (C₁₈, 5 μm, 250 × 4.6 mm) column using isocratic condition, with acetonitrile, 5 mM disodium hydrogen phosphate buffer adjusted to pH 5, and methanol (50:25:25, v/v/v) as mobile phase. The flow rate for analysis was 1.0 mL min⁻¹, and the selected chromatographic conditions effectively separated moxifloxacin hydrochloride and difluprednate with retention time of 3.6 and 6.6 min, respectively, at a detection wavelength of 254 nm. Chromatographic development in HPTLC was performed on precoated silica gel 60F₂₅₄ aluminium plates with n-hexane, 6 M ammonia, and acetone (5:1.8:2, v/v/v) as mobile phase. The detection wavelength for simultaneous estimation of both drugs was 232 nm in HPTLC, and the Rf values for moxifloxacin hydrochloride and difluprednate were 2.2 and 7.1, respectively. The linear concentration range for HPLC method was 5 to 50 μg mL⁻¹ and 1 to 10 μg mL⁻¹; and for HPTLC method was 1200 to 2200 ng band⁻¹ and 200 to 1200 ng band⁻¹ for moxifloxacin hydrochloride and difluprednate, respectively. Moreover, Bartlett's test applied on the calibration peak areas revealed homoscedasticity of variance for both the methods. Both methods were validated with respect to system suitability, specificity, linearity, precision, accuracy, and robustness. The mean percentage recoveries for marketed formulation in terms of accuracy were found to be 100.53 and 100.58 for HPLC; and 100.56 and 100.30 for HPTLC for moxifloxacin hydrochloride and difluprednate, respectively. The pooled percent relative standard deviation (% RSD) value for repeatability, intermediate precision, accuracy, and robustness studies for both the methods were found to be less than 2. Result of paired t-test at 95% confidence level reveals that there is no significant difference between recoveries of drugs, using both methods. The results of the developed chromatographic methods were acceptable assuring that these methods can be successfully applied for routine quality control testing of both bulk and ophthalmic dosage forms, without any interference from the excipients.

Key Words: moxifloxacin hydrochloride, difluprednate, HPLC, HPTLC, validation
Introduction

Moxifloxacin, a fourth generation 8-methoxy fluoroquinolone derivative, chemically is 1-cyclopropyl-6-fluoro-1, 4-dihydro-8methoxy-7-[(4aS, 7aS-octa-hydro-6H-pyrrolol (3,4b) pyridin6-yl)]-4-oxo-3-quinoline carboxylic acid, and monohydrochloride (Fig. 1a) with extended-spectrum and improved activity against Gram positive bacteria (staphylococci, streptococci, enterococci), anaerobes, and atypical bacteria. Moxifloxacin as an antibiotic is used to treat respiratory infections, including acute sinusitis, acute exacerbations of chronic bronchitis, and community-acquired pneumonia, as well as dermatological infections, as a second-line agent in tuberculosis [1–3]. Difluprednate, a synthetic glucocorticoid, chemically is DFBA, 6a,9-difluoro-11b,17,21-trihydroxypregna-1,4-diene-3,20-dione-17-butyrate-2-acetate (Fig. 1b), and is used for the treatment of inflammation and pain associated with ocular surgery. Corticosteroids act by the induction of phospholipase A2 inhibitory proteins, lipocortins [4, 5].

![Chemical structure of (A) moxifloxacin hydrochloride and (B) difluprednate](image)

Fig. 1. Chemical structure of (A) moxifloxacin hydrochloride and (B) difluprednate

Most of the analytical methods published for the determination of moxifloxacin hydrochloride are based on spectrophotometry, reversed phase high-performance liquid chromatography (RP-HPLC), and high-performance thin-layer chromatography (HPTLC) [6–11], and an official assay for moxifloxacin ophthalmic solution based on HPLC is reported in United States Pharmacopeia (USP) [12], while only one method is reported in literature for estimation of difluprednate by semi-micro HPLC method [13]. However, none of the research papers that report analytical method for estimation of combination of both moxifloxacin hydrochloride and difluprednate ophthalmic formulation were available. In context to this, chromatographic methods [14–16] that find wide application in research
and industry were developed for the determination of moxifloxacin hydrochloride and difluprednate in ophthalmic formulation. Hence, the present manuscript is the first one that describes the development of a new RP-HPLC method and HPTLC method, both of which are validated as per International Conference on Harmonization (ICH) guidelines (Q2R1), meeting the required criteria for specificity, accuracy, and precision, and are suitable for routine quality control analysis of both drugs in ophthalmic dosage form.

**Experimental**

**Materials and Reagents**

Moxifloxacin hydrochloride and difluprednate 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 moxifloxacin and 0.5 mg of difluprednate per each milliliter (Ajanta Pharma Limited, Mumbai) was procured from local market. Methanol of analytical reagent (AR) grade was procured from SDFCL, Mumbai. HPLC grade disodium hydrogen orthophosphate, acetonitrile, methanol, orthophosphoric acid, and triethylamine were procured from Merck Specialities Private Limited. Double distilled water was prepared by using quartz distillation assembly.

**Instrumentation**

HPLC system, with LC solutions data handling system (Shimadzu-LC2010-CHT), with PDA detector and an auto sampler was used for the analysis. The data was recorded using LC 2010 solutions software version 1.25. A Camag HPTLC system (Switzerland) comprising of Camag Linomat V applicator; Camag TLC scanner IV; ultraviolet (UV) cabinet with dual wavelength UV lamp; Camag flat bottom and twin trough chamber (10 × 10 cm); Camag winCATS version 1.4.6 software; Hamilton syringe, 100 μL (Linomat syringe 659.0014, Hamilton-Bonaduz Schweiz, Camag, Switzerland); and precoated silica gel 60F254 aluminium plates, 10 × 10 cm, 100 μm thickness (Merck, Darmstadt, Germany) were used during the study.
Preparation of Stock and Working Standard Solution

**HPLC**

A stock solution of moxifloxacin (MOXI, 1000 µg mL$^{-1}$) and difluprednate (DIFLU, 200 µg mL$^{-1}$) was prepared by dissolving an accurately weighed quantity in mobile phase. Further, final concentration of MOXI (100 µg mL$^{-1}$) and DIFLU (20 µg mL$^{-1}$) by tenfold dilution of the stock solution with mobile phase was prepared.

**HPTLC**

A stock solution of MOXI (1000 µg mL$^{-1}$) and DIFLU (1000 µg mL$^{-1}$) was prepared by dissolving an accurately weighed quantity in methanol. Further, final concentration of MOXI (100 µg mL$^{-1}$) and DIFLU (100 µg mL$^{-1}$) was prepared by tenfold dilution of the stock solution with methanol.

**HPLC Analysis**

The chromatographic determination was performed on a reversed-phase stainless steel column, filled with octadecylsilane chemically bonded to porous silica particles (Inertsil C$_{18}$, 5 µ, 150 mm × 4.6 mm) with the mobile phase containing 5 mM disodium hydrogen orthophosphate buffer adjusted to pH 5.0, acetonitrile, and methanol (25:50:25, v/v/v). The mobile phase was prepared daily, filtered using 0.45 µm nylon filter, and degassed in sonicator for 10 min before use. The flow rate was adjusted to 1.0 mL min$^{-1}$, and the elution was monitored at 254 nm. The injection volume to carry out chromatography was set as 10 µL, and mobile phase was used as a diluent.

From working standard solution, aliquot of 0.5–5 mL was withdrawn and further diluted to 10 mL with mobile phase to obtain a concentrations range of 5 to 50 µg mL$^{-1}$ of MOXI and 1 to 10 µg mL$^{-1}$ of DIFLU, respectively. Ten microliters of the different concentration of standard solution of MOXI and DIFLU were injected according to optimized conditions, and all measurements were repeated five times. A calibration curve of area vs. concentration (µg mL$^{-1}$) was plotted, and regression analysis was performed.

**HPTLC Analysis**

The solutions were spotted in the form of bands having band width 6 mm with a microsyringe on precoated silica gel aluminium Plate 60F$_{254}$, using a
Camag Linomat 5 sample applicator. Linear ascending development was carried out in twin trough glass chamber. Mobile phase components were mixed prior to use, and the development chamber was left to saturate with mobile phase vapor for 20 min before each chromatographic run. The mobile phase consisted of n-hexane–6 M ammonia–acetone (5:1.8:2, v/v/v). The length of chromatographic run was 80 mm, and all measurements were made in the reflectance–absorbance mode at 272 nm; slit dimension of 6.00 × 0.30 mm, micro; scanning speed of 20 mm s⁻¹; and data resolution of 100 μm step⁻¹. The source of radiation was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm.

Standard solutions ranging from 12.0 to 22.0 μL for MOXI and 2.0 to 12.0 μL for DIFLU were applied on HPTLC plate that gave a final concentration range of 1200 to 2200 ng band⁻¹ for MOXI and 200 to 1200 ng band⁻¹ for DIFLU, respectively. Each concentration was spotted five times on HPTLC plate. Calibration curve was plotted as peak areas versus corresponding concentrations.

Analysis of Ophthalmic Formulation by HPLC Analysis

An accurately measured volume of eye drops equivalent to 25 mg of MOXI and 2.5 mg of DIFLU was transferred into 25 mL volumetric flask and diluted with 20 mL mobile phase. The mixture was sonicated for 15 min and further dilution up to the mark with mobile phase, followed by filtration through Whatman filter paper no. 42 wetted with mobile phase. Further dilution with mobile phase was performed to obtain a concentration of 40 μg mL⁻¹ of MOXI and 4 μg mL⁻¹ of DIFLU. The method described above was then applied for determination of peak area, and triplicate analysis was performed by following the same procedure.

Analysis of Ophthalmic Formulation by HPTLC Analysis

An accurately measured sample volume equivalent to 5 mg of MOXI and 0.5 mg of DIFLU was transferred into a 25-mL volumetric flask containing 20 mL methanol, followed by sonication for 15 min and further dilution up to 25 mL with methanol. The resulting solution was filtered through Whatman filter paper No. 42 wetted with methanol. Ten microliter of the filtered solution (2000 ng of MOXI and 200 ng of DIFLU per band) was applied on the HPTLC plate followed by development and scanning; the analysis was repeated in triplicate as described by above method.
Validation

The method was validated in accordance with ICH guidelines Q2 (R1) for evaluation of various parameters: linearity, precision, accuracy, LOD, LOQ, specificity, and robustness for both methods [17].

System suitability in HPLC was determined at a concentration of 30 μg mL\(^{-1}\) of MOXI and 6 μg mL\(^{-1}\) of DIFLU using the stock solution by six replicate injections. The linearity of both methods was evaluated by linear regression analysis in the concentration range of 5 to 50 μg mL\(^{-1}\) (MOXI) and 1 to 10 μg mL\(^{-1}\) (DIFLU) for HPLC, and 1200 to 2200 ng band\(^{-1}\) (MOXI) and 200 to 1200 ng band\(^{-1}\) (DIFLU) for HPTLC in terms of correlation coefficient. Furthermore, Bartlett's test was applied on calibration data for evaluation of homoscedasticity of variance [18]. The detection limit and quantification limit were determined from calibration curves according to ICH guidelines for both the methods. Specificity of HPLC and HPTLC method was ascertained by comparing standard drug and sample formulation. Peak purity of MOXI and DIFLU was also assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M), and peak end (E) positions of the band.

Precision was evaluated by performing repeatability and intermediate precision for both methods using a concentration of 30.00 μg mL\(^{-1}\) of MOXI and 6.00 μg mL\(^{-1}\) of DIFLU, respectively. Three replicates of three concentrations for each drug were analyzed for repeatability, and three replicates of three concentration for each drug were analyzed on different days to ascertain intermediate precision, and results were expressed as percent relative standard deviation (% RSD). In HPTLC, repeatability was carried out by performing three replicates of three different concentrations (1400 ng, 1800 ng, and 2200 ng for MOXI; 200 ng, 800 ng, and 1200 ng for DIFLU), and peak area measured was expressed in terms of % RSD. To demonstrate the accuracy of the proposed methods, recovery studies were carried out by standard addition method. Freshly prepared solution of ophthalmic formulation was spiked with 50%, 100%, and 150% concentration level of standard. Recovery studies in HPTLC were carried out by spiking three different amounts of moxifloxacin standard (600 ng, 1200 ng, and 1800 ng) to the dosage form (1200 ng band\(^{-1}\)) and difluprednate standard (200 ng, 400 ng, and 600 ng) to the dosage form (400 ng band\(^{-1}\)) by standard addition method.

Method robustness was performed by changing the flow rate, wavelength, and mobile phase composition to evaluate the impact on the performance of the method. For the robustness study, small deliberate changes of various factors in HPLC were the following: flow rate, 1.1 mL min\(^{-1}\) and 0.9 mL min\(^{-1}\); wavelength, 252 nm and 256 nm; and mobile phase...
composition, acetonitrile:buffer:methanol (50:24:26, \( v/v/v \), and 51:24:25, \( v/v/v \)). In HPTLC, the effect of deliberate variations in method parameters was the composition of the mobile phase of n-hexane:6 M ammonia:acetone (4.8:1.8:1.8, \( v/v/v \), and 5.2:1.8:2.2, \( v/v/v \)), saturation time (15 min and 25 min), development distance (7 cm and 9 cm), and wavelength scan (270 nm and 274 nm). The effect of these changes on the \( R_f \) values, \( R_f \) value and peak areas was evaluated by calculating the % RSD for each parameter.

The standard solution of MOXI and DIFLU was stored, unprotected from light, at ambient condition and assayed after 0 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h against a freshly prepared standard solution.

**Results and Discussion**

**Chromatographic Development**

In HPLC method, various trials were performed using polar solvents, acetonitrile and methanol, with and without addition of buffer for optimization of mobile phase. Among these preliminary trials, acetonitrile with methanol in the ratio of 50:50 produced retention time at 3.2 for MOXI but DIFLU did not elute out of the column. Thus, acetonitrile was combined with phosphate buffer in the ratio of 70:30 where, again, retention time for MOXI was 3.8 but DIFLU did not elute out. Preliminary trials showed that retention time of MOXI was not greatly affected by changing polarity with acetonitrile and methanol, and peak for DIFLU appeared in the chromatogram by using disodium hydrogen orthophosphate buffer instead of phosphate buffer. Hence, further disodium hydrogen orthophosphate buffer 5mM in combination with acetonitrile in the ratio of 50:50 produced good peak shape for both drugs, but retention time of DIFLU was too high, i.e., 19.1 min, and that of MOXI, 3.8 min. Hence, further trial was focused on decreasing the retention time of DIFLU by decreasing the volume of acetonitrile. In context to this, acetonitrile, disodium hydrogen orthophosphate buffer, and methanol, when tried in ratio of 50:25:25, produced peaks for MOXI and DIFLU at retention time of 3.7 and 6.8 min, but peak shape was not proper for both drugs (fronting in peak for MOXI and broad peak for DIFLU). Finally, the best results were obtained by adjusting the pH of buffer and the final mobile phase composition optimized was a mixture of 5 mM disodium hydrogen orthophosphate adjusted to pH 5.0 with orthophosphoric acid, acetonitrile, and methanol in the ratio of 25:50:25. Under these optimized chromatographic conditions, the retention time of MOXI and DIFLU was 3.6 and 6.6 min, respectively, at 254 nm (Fig. 2A and B).
Various trials were performed for the optimization of mobile phase in HPTLC method, where toluene-n-hexane and methanol in the ratio of 5:5 (v/v) did not produce good peak shape with MOXI retained at its original application position. Hence, ammonia as a modifier was added which gave acceptable $R_f$ value for MOXI, but $R_f$ value of DIFLU was very high, with
improper peak shape. Hence, acetone was added further to improve peak shape of both drugs showing acceptable $R_f$ value. The proportion of mobile phase was then adjusted using n-hexane, 6 M ammonia and acetone. Finally, the optimized mobile phase consisted of n-hexane, 6 M ammonia, and acetone (5:1.8:2, $v/v/v$) showing good resolution without any fronting. Under these optimized chromatographic conditions, the $R_f$ of MOXI and DIFLU at 272 nm was 0.22 and 0.71, respectively (Fig. 3A and B).

Validation

Various method validation parameters: linearity, precision, accuracy, LOD, LOQ, specificity, and robustness evaluated in accordance with ICH guidelines Q2 (R1) for both the proposed methods are as described below.

System suitability testing in HPLC method showed that the method was suitably performed under the optimized conditions, and % RSD was found less than 2%, for system suitability parameters: $R_t$ (for MOXI, 3.602 ± 0.001; for DIFLU, 6.586 ± 0.002), area (for MOXI, 266392.6 ± 117.167; for DIFLU, 58067 ± 50.105), tailing factor (less than 1.25), number of theoretical plates (more than 5000), and resolution (10.62 ± 0.064) (Fig. 4).

![System suitability chromatogram for MOXI standard, 30 μg mL$^{-1}$, and DIFLU standard, 6 μg mL$^{-1}$, showing $R_t$ of 3.6 min for MOXI and $R_t$ of 6.6 min for DIFLU](image)

**Fig. 4.** System suitability chromatogram for MOXI standard, 30 μg mL$^{-1}$, and DIFLU standard, 6 μg mL$^{-1}$, showing $R_t$ of 3.6 min for MOXI and $R_t$ of 6.6 min for DIFLU

**Linearity**

For HPLC method, the calibration curve was found to be linear over the concentration range of 5 to 50 μg mL$^{-1}$ for MOXI and 1 to 10 μg mL$^{-1}$ for DIFLU, and for HPTLC method, the calibration curve was found to be
linear over the concentration range of 1200 to 2200 ng band\(^{-1}\) for MOXI and 200 to 1200 ng band\(^{-1}\) for DIFLU. The linearity of calibration graphs and adherence of the system to Beer's law were validated by high value of correlation coefficient, and no significant difference was observed in the slopes of standard curves (ANOVA; \(P < 0.05\)). Further, homoscedasticity of variance on the data of linearity and peak area with respect to concentration range for both methods for MOXI and DIFLU were also evaluated and validated by Bartlett's test. 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 (Table I).

Table I. Analytical validation parameters for the proposed HPLC and HPTLC method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Moxifloxacin</th>
<th>Difluprednate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC</td>
<td>HPTLC</td>
</tr>
<tr>
<td>Linearity range(^{a,b})</td>
<td>5 to 50</td>
<td>1200 to 2000</td>
</tr>
<tr>
<td>Correlation coefficient ((r^2))</td>
<td>0.9996</td>
<td>0.9991</td>
</tr>
<tr>
<td>Slope (\pm SD (S_b))</td>
<td>7926.163 (\pm 0.925)</td>
<td>3.1602 (\pm 0.0213)</td>
</tr>
<tr>
<td>Confidence limit of slope</td>
<td>7926.496 to 7925.829</td>
<td>3.1678 (\pm 3.1525)</td>
</tr>
<tr>
<td>Intercept (\pm SD (S_a))</td>
<td>30662.905 (\pm 68.815)</td>
<td>1567.709 (\pm 31.901)</td>
</tr>
<tr>
<td>Confidence limit of intercept</td>
<td>30686.88 to 30637.31</td>
<td>1579.20 (\pm 1556.22)</td>
</tr>
<tr>
<td>Limit of detection(^b)</td>
<td>0.029</td>
<td>33.31</td>
</tr>
<tr>
<td>Limit of quantitation(^b)</td>
<td>0.087</td>
<td>100.95</td>
</tr>
<tr>
<td>Bartlett's test ((\chi^2))</td>
<td>1.31 (\times 10^{-6})</td>
<td>0.0081</td>
</tr>
<tr>
<td>Precision(^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability</td>
<td>0.02–0.12</td>
<td>0.39–0.98</td>
</tr>
<tr>
<td>Interday precision</td>
<td>0.08–0.25</td>
<td>1.10–1.70</td>
</tr>
<tr>
<td>Accuracy(^e)</td>
<td></td>
<td></td>
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<tr>
<td>50%</td>
<td>99.72 (\pm 1.48)</td>
<td>99.23 (\pm 0.01)</td>
</tr>
<tr>
<td>100%</td>
<td>100.36 (\pm 0.71)</td>
<td>100.36 (\pm 0.02)</td>
</tr>
<tr>
<td>150%</td>
<td>101.10 (\pm 0.24)</td>
<td>100.37 (\pm 0.01)</td>
</tr>
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</table>

\(^a\)Average of five determinations.
\(^b\)\(\mu g\) mL\(^{-1}\) for HPLC and ng band\(^{-1}\) for HPTLC method.
\(^c\)Calculated value less than tabulated value, 9.488 at 95% confidence interval.
\(^d\)Average of three determinations for each concentration.
\(^e\)Average of three determinations at each level.

**Precision**

For HPLC and HPTLC proposed methods, the % RSD values in Table I show that the proposed methods provide acceptable intra-day and inter-day variation of MOXI and DIFLU, thus, indicating acceptable repeatability and reproducibility of the developed method. The repeatability of sample
application and measurement of peak area were expressed in terms of % RSD and were found to be less than 2% for both MOXI and DIFLU in HPTLC method.

**Accuracy**

Both proposed methods when used for estimation of both drugs from pharmaceutical dosage form after spiking with standard drug, at three concentration levels 50, 100, and 150 %, afforded acceptable percent recovery of 99.23% to 100.74% with % RSD less than 2, revealing that the proposed methods are suitable for the analysis of bulk and formulation (Table I).

**Robustness Study**

For HPLC method, typical variations in liquid chromatography conditions were used to evaluate the robustness of the assay method. The robustness acceptance criteria set in the validation were the same as that of established on system suitability test described above, and the results, presented in Table II, indicate that the selected factors remained unaffected by small variations of these parameters. In HPTLC method, deliberate change in different parameters like mobile phase composition, saturation time, development distance, wavelength scan, time from spotting to chromatography, and mobile phase volume showed % RSD of peak area.

*Table II. Robustness study for HPLC and HPTLC proposed methods*

<table>
<thead>
<tr>
<th>Method</th>
<th>Parameters</th>
<th>RSDs (%)</th>
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<tr>
<td></td>
<td></td>
<td>MOXI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIFLU</td>
</tr>
<tr>
<td>HPLC</td>
<td>Change in flow rate (1.0 mL min⁻¹ ± 0.1)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Change in mobile phase buffer pH change (5 ± 0.2)</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Change in change mobile phase ratio (25:50:25 ± 2)</td>
<td>0.35</td>
</tr>
<tr>
<td>HPTLC</td>
<td>Mobile phase composition (5 ± 0.2:1.8:2 ± 0.2)</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Saturation time (20 min ± 5 min)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Development distance (8 cm ± 1 cm)</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Wavelength (± 2 nm)</td>
<td>0.43</td>
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<th>RSDs (%)</th>
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<tbody>
<tr>
<td>MOXI</td>
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<td>DIFLU</td>
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</table>

*Average of three determinations for concentrations; HPLC method: 30.00 μg mL⁻¹ MOXI and 6.00 μg mL⁻¹ DIFLU; HPTLC method: 1400, 1800, and 2200 ng band⁻¹ for MOXI, and 200, 800, and 1200 ng band⁻¹ for DIFLU*
less than 2%, indicating that the method is robust (Table II). Moreover, insignificant differences in peak areas and less variability in retention time and retention factor were observed.

**Specificity**

For RP-HPLC, $R_t$ for both drugs appeared at same position in the chromatogram of ophthalmic dosage formulation when compared with standard without any additional peak of interference or excipient. The average retention times for MOXI and DIFLU in ophthalmic dosage form were found to be 3.6 and 6.6 min, respectively, for three replicates (Fig. 5A).

For HPTLC method, the peak purity of both drugs in ophthalmic formulations when evaluated by comparing the overlaid spectra at peak start, peak apex, and peak end positions of the spot showed good correlation, i.e., $r(S, M) = 0.9997$ and $r(M, E) = 0.9998$, indicating specificity in presence of excipients (Fig. 5B). Moreover, the chromatogram of the pharmaceutical formulation using the developed method showed only two peaks at $R_t$ value of 0.22 and 0.71 for moxifloxacin and difluprednate, respectively, which was found to be at the same $R_t$ value for both standard drugs by comparison of chromatograms.

*Fig. 5. Specificity study showing (A) HPLC chromatogram of ophthalmic dosage form (2000 ng band$^{-1}$ of MOXI and 200 ng band$^{-1}$ of DIFLU compared with standard, 1800 ng band$^{-1}$ of MOXI and 800 ng band$^{-1}$ of DIFLU); (B) HPTLC overlain peak purity spectra of ophthalmic dosage form with standard drug*
Analysis of Ophthalmic Formulation

Experimental results for analysis of ophthalmic formulation, expressed as percentage of label claim, were in good agreement with the label claims, thereby suggesting no interference from any excipients by both methods (Table III). The low % RSD value indicated the suitability of these proposed methods for routine analysis of ophthalmic formulation containing MOXI and DIFLU.

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Drug</th>
<th>Label claim</th>
<th>% Mean amount of drug ± % RSD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MOXI</td>
<td>5 mg mL⁻¹</td>
<td>100.58 ± 0.143 100.56 ± 0.207</td>
</tr>
<tr>
<td></td>
<td>DIFLU</td>
<td>0.5mg mL⁻¹</td>
<td>100.53 ± 0.057 100.30 ± 0.173</td>
</tr>
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</table>

*aAverage of three determinations.

HPTLC versus HPLC

Statistical evaluation was performed using Student's t-test at 95% confidence level (P = 0.05) on the data for analysis of ophthalmic formulation. To test means, a paired t-test was applied to test the differences between the proposed HPTLC and HPLC method. The test removes any variations between samples. The obtained value of tstat for moxifloxacin hydrochloride (0.1015) and difluprednate (2.6457) is lower than two tail tcrit (4.3027), which leads to the conclusion that there is no significant difference between the means.

Conclusion

The proposed HPTLC and HPLC methods provide simple, accurate, and reproducible quantitative analysis for simultaneous determination of moxifloxacin hydrochloride and difluprednate in ophthalmic formulation. Both methods were validated as per ICH guidelines. Statistical analysis indicates no significant difference for analysis of formulation for both methods, and thereby the proposed HPTLC and HPLC methods appear to be equally suitable for routine determination of moxifloxacin hydrochloride and difluprednate simultaneously in pharmaceutical formulation.
References


Accepted by DA