Original Article

Validated high-performance thin-layer chromatographic (HPTLC) method for simultaneous determination of nadifloxacin, mometasone furoate, and miconazole nitrate cream using fractional factorial design

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ABSTRACT

A high-performance thin-layer chromatographic method for simultaneous determination of nadifloxacin, mometasone furoate, and miconazole nitrate was developed and validated as per International Conference on Harmonization guidelines. High-performance thin-layer chromatographic separation was performed on aluminum plates precoated with silica gel $60F_{254}$ and methanol:ethyl acetate:toluene: acetonitrile:3M ammonium formate in water (1:2.5:6.0:0.3:0.2, % v/v) as optimized mobile phase at detection wavelength of 224 nm. The retardation factor (R_f) values for nadifloxacin, mometasone furoate, and miconazole nitrate were 0.23, 0.70, and 0.59, respectively. Percent recoveries in terms of accuracy for the marketed formulation were found to be 98.35—99.76%, 99.36—99.65%, and 99.16—100.25% for nadifloxacin, mometasone furoate, and miconazole nitrate, respectively. The pooled percent relative standard deviation for repeatability and intermediate precision studies was found to be < 2% for three target analytes. The effect of four independent variables, methanol content in total mobile phase, wavelength, chamber saturation time, and solvent front, was evaluated by fractional factorial design for robustness testing. Amongst all four factors, volume of methanol in mobile phase appeared to have a possibly significant effect on retention factor of miconazole nitrate compared with the other two drugs nadifloxacin and mometasone furoate, and therefore it was important to be carefully controlled. In summary, a novel, simple, accurate, reproducible, and robust high-performance thin-layer chromatographic method was developed, which would be of use in quality control of these cream formulations.

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1. Introduction

Nadifloxacin (ND), chemically (RS)-9-fluoro-8-(4-hydroxy-piperidin-1-yl)-5-methyl-1-oxo-6,7-dihydro-1H,5H-pyrido [3,2,1-ij] quinoline-2-carboxylic acid (Figure 1A), is a potent antibacterial drug. ND has not yet been officially described in any pharmacopoeia. Mometasone furoate (MF), a glucocorticoid, chemically 9α,21-dichloro-llβ-hydroxy-16α-methyl 3,20-dioxopregna-l,4-dien-17-yl furan-2-carboxylate (Figure 1B), is used for antiinflammatory and antipruritic properties [1,2]. Miconazole nitrate (MN), is an antifungal drug, chemically known as (RS)-1-[2-(2,4dichlorophenylmethoxy)-2-(2,4-dichlorophenyl)ethyl] lH-imidazole nitrate (Figure 1C). It is used to exhibit a broad spectrum of antimicrobial activity for systemic and local treatment of vaginal and topical fungal infections [3]. A combination of all these three drugs available as a cream has been used for the treatment of dermatoses topically.

A literature survey revealed various stabilities, indicating reverse-phase high-performance liquid chromatography (HPLC) and spectrophotometric methods for MF and MN, and HPLC and high-performance thin-layer chromatographic (HPTLC) methods for all three drugs individually and in combination with other drugs [4—23]. Spectrophotometric and HPTLC methods for all three drugs individually and in combination for simultaneous estimation have also been reported [24—29]. However, development of a HPTLC method for simultaneous estimation of ND, MF, and MN in combined dosage form has not been reported.

Recently, HPTLC is widely employed for the quantification of drugs because of low maintenance cost, lower analysis time, low mobile phase consumption per sample, and need for minimal sample clean-up. It facilitates automated application of samples and scanning of plates and, moreover, HPTLC as method recently has been proposed to be included in various pharmacopoeia [30—33].

Analytical quality by design (AQbD) is a systematic approach of method development that begins with predefined objectives and emphasizes method understanding and its performance, based on sound science and quality risk management [34]. The main objective of AQbD is to reduce variations in the measurements by controlling various factors that

affect method performance thereby resulting in less variation in interlaboratory studies and assuring reproducibility. Design of experiment (DoE) is an integral part of AQbD that includes use of experimental design, mathematical model generation by ANOVA analysis, and graphical representations, showing correlation between factors and response [35—38]. Therefore, design of experimentation is required to study the effect of previously identified factors affecting the method and defining a robust AQbD design space where the method can be operated anywhere in that region. Method transfer and reproducibility in interlaboratory studies are the potential benefits of AQbD [39—40].

This research article focuses on the determination of robustness of HPTLC analytical method by fractional factorial design (FFD). Among the various experimental designs, FFD as a response surface was preferred for prediction of nonlinear response and also due to its flexibility, in terms of experimental runs and information related to the factor's main and interaction effects. Therefore a novel, simple, accurate, reproducible HPTLC method was developed for simultaneous estimation of ND, MF, and MN in pharmaceutical dosage form, using FFD design for robustness testing. Therefore, this research paper describes the development of HPTLC method for simultaneous estimation of ND, MF, and MN using the DoE approach for method validation.

# 2. Materials and methods

2.1. Materials

Working standards of ND and MN were kindly provided as a gratis sample from Hetero Drugs Limited, Hyderabad, India and MF from Cipla Ltd., Mumbai, India. All solvents and chemicals used were purchased from Merck Specialities Pvt. Ltd., India. Marketed cream formulation; Bactimax cream (Ajanta Pharma Ltd., Mumbai) used in this study was procured from the local market.

2.2. Instrumentation

Linomat 5 applicator (Camag, Switzerland), twin trough chamber (20 × 10 cm; Camag, Switzerland), TLC scanner IV (Camag, Switzerland), win CATS version 1.4.6 software (Camag, Switzerland), Microsyringe (Linomat syringe 659.0014, Hamilton—Bonaduz Schweiz, Camag, Switzerland), UV chamber (Camag, Switzerland), precoated silica gel 60F$_{254}$ aluminium plates (20 × 10 cm, 100 μm thickness; Merck, Darmstadt, Germany) were used in the study.

2.3. Preparation of standard solutions

A stock solution of ND, MF, and MN was prepared separately by weighing accurately 10 mg of drug followed by dissolution in methanol in a 100-mL volumetric flask and dilution up to the mark with methanol, to obtain a concentration of 100 μg/mL. This stock solution was appropriately diluted with methanol to obtain a working standard solution for ND, MF, and MN.
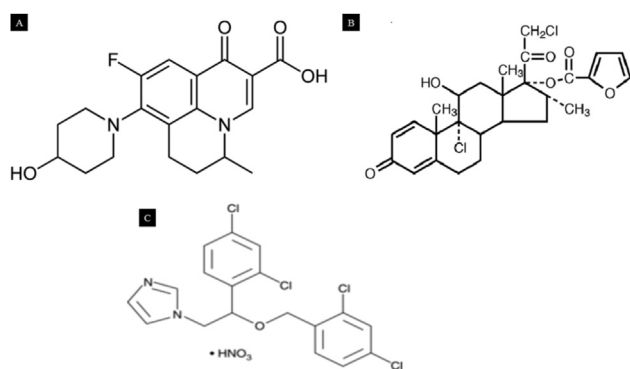
Figure 1 — (A) Chemical structures of nadifloxacin; (B) mometasone furoate; (C) miconazole nitrate.

2.4. Chromatographic development and scanning

Suitable volumes of standard and sample solutions were applied to the HPTLC plates, 10 mm from the bottom and 15 mm from the side edges in the form of bands with band length of 6 mm on precoated silica gel aluminum plate 60F$_{254}$, (10 × 10 cm) 100 μm thickness; using a Camag Linomat V sample applicator. The mobile phase consisted of methanol:ethyl acetate:toluene:acetonitrile:3M ammonium formate in water (1:2.5:6.0:0.3:0.2, % v/v) and the length of chromatographic run was 8.5 cm. Mobile phase components were mixed prior to use and the development chamber was left to saturate with mobile phase vapor for 20 minutes before each run. Development was carried out by the ascending technique to a migration distance of 85 mm. TLC plates were then dried in a current of air with an air dryer. Densitometric scanning was performed and all measurements were made in the reflectance absorbance mode at 224 nm, slit dimension (6.0 × 0.30 mm, micro), scanning speed 20 mm/s, data resolution 100 μm/step, optical filter (second order), filter factor (Savitsky golay 7). The source of radiation was deuterium lamp emitting a continuous UV spectrum between 200 nm and 400 nm. Concentration of the drug was determined from the intensities of diffusely reflected lights. Evaluation was via peak areas with linear regression analysis.

2.5. Method validation

The method was validated in accordance with ICH guidelines Q2 (R1) for evaluation of various parameters that include linearity, precision, accuracy, limit of detection, limit of quantitation, specificity, and robustness [41]. Linear relationship between peak area and concentration of all three drugs were evaluated by making five replicate measurements in the concentration range, 400–2400 ng/band for ND and MN, and 100–600 ng/band for MF respectively. Calibration plots were constructed using the method of ordinary regression analysis for checking linearity. Homoscedasticity of variance was also evaluated for the response by Bartlett's test. Precision of the developed method was evaluated by performing repeatability and intermediate precision studies. Repeatability on same day and intermediate precision on different days was carried out by performing three replicates of three different concentrations (800 ng, 1600 ng, and 2400 ng) of ND and MN and (200 ng, 400 ng, and 600 ng) of MF. The analysis was repeated in triplicate and %RSD was calculated for peak area. Accuracy of the method was ascertained by performing recovery at three levels (50%, 100%, and 150%). Recovery studies were carried out by spiking three different amounts of ND, MN, (400 ng, 800 ng, and 1200 ng) and MF standard (100 ng, 200 ng, and 300 ng) to the dosage form for ND and MN (800 ng/band) and for MF (200 ng/band) by standard addition method. Recovery studies were performed in triplicate. As per ICH guidelines, 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 ND, MF, and MN using the formula, limit of detection = 3.3 * σ/S; limit of quantitation = 10 * σ/S where, "σ" is standard deviation of response; and "S" is slope of calibration curve. The specificity of the method was ascertained by analyzing peak purity of

standard drug and cream formulation. The spot for ND, MF, and MN in sample was confirmed by comparing the R_f and spectra of all the three drugs with that of standard. The peak purity of each of the three drugs was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M), and peak end (E) position of the spot.

The robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters. To study robustness, fractional factorial design (FFD) was applied; four factors half fractional design (2^{4-1}). In the present study, four factors were selected based on the criticality of factors observed during trial runs, chromatographic intuition and experience gained from previous studies, volume of methanol in the mobile phase composition (A), chamber saturation time (B), wavelength (C), and solvent front (D). To quantitatively analyze the deviation of the considered response, R_f from the original value, the ranges of factors examined were deliberately changed from the optimum method settings of all three drugs. The four factors with their deliberate changes in terms of high and low level are as shown in Table 1. All experiments were performed in randomized order to minimize the bias effects of uncontrolled factors according to the experimental domain of the selected variables. The experiments were performed based on the experimental domain and the responses were recorded in the form of retention factor of ND, MF, and MN to check the robustness of the method.

2.6. Analysis of marketed formulation

Marketed formulation (Bactimax cream), an accurately measured amount of cream (0.5 g) equivalent to 1.0% w/w of ND, 0.1% w/w of MF, and 2.0% w/w of MN was transferred into 100 mL volumetric flask followed by addition of 30 mL methanol. The solution was sonicated for 20 minutes and the volume was made up to the mark with methanol and again sonicated for 10 minutes. The solution was filtered using whatman paper 0.45 μm, and 1 mL was further diluted to 10 mL with methanol. The resultant sample solution was used for chromatographic development and scanning followed by analysis. The analysis was repeated in triplicate.

2.7. Statistical analysis

All data analysis of experimental design was performed by using the Design-Expert crack version 7.0.0 (Stat-Ease Inc., Minneapolis, USA) and remaining statistical calculations were performed by use of Microsoft Excel 2013 software (Microsoft Corporation, USA). Bartlett's test and test for lack of fit were

Table 1 – Experimental factors and levels used in FFD.

Factors	High level	Low level
Methanol volume in mobile phase composition (mL) (A)	1.2	0.8
Chamber saturation time (min) (B)	22	18
Wavelength (nm) (C)	226	222
Solvent front (cm) (D)	8.7	8.5
FFD = fractional factorial design.		
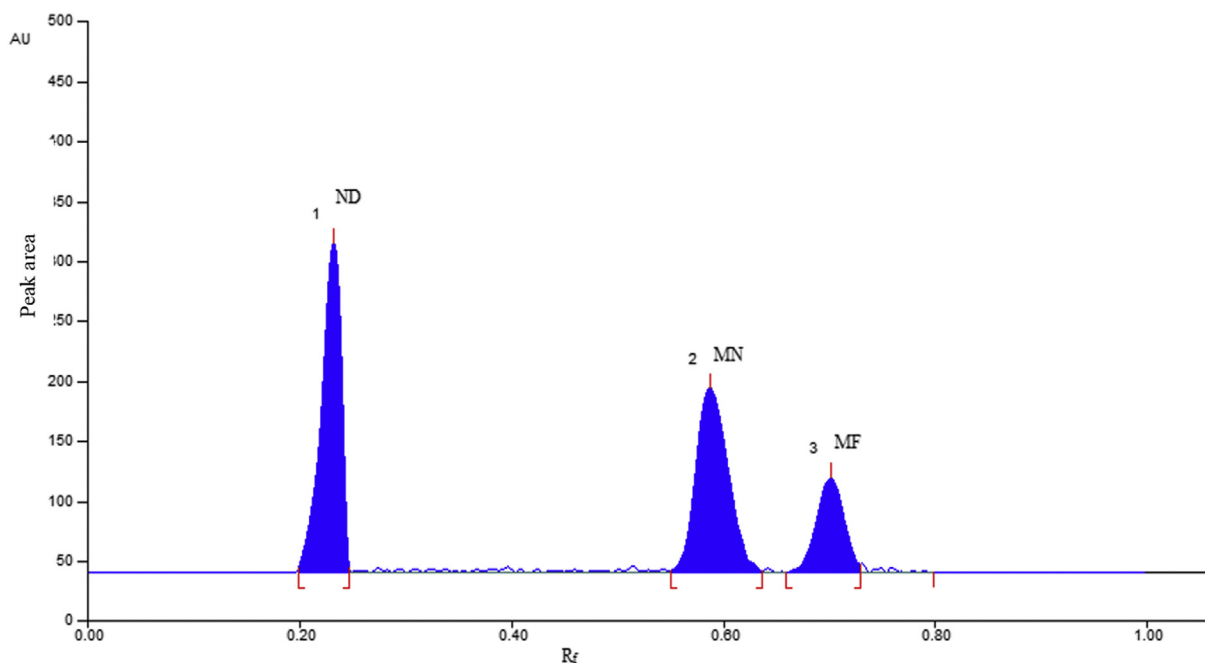
Figure 2 – TLC chromatogram of standards: ND (R_f 0.23), MF (R_f 0.70), and MN (R_f 0.59). MF = mometasone furoate; MN = miconazole nitrate; ND = nadifloxacin; TLC = thin layer chromatography.

applied on the data of areas of linearity for evaluation of homoscedasticity of variance and deviation from linearity [42].

3 Result and discussion

The common detection wavelength selected for analysis was 224 nm as all three drugs were showing optimum response at 224 nm. Mobile phase optimization was carried out in different solvent systems and different ratios of various solvents were tried such as n-hexane, toluene, methanol, ethyl acetate, acetonitrile, diethyl ether, chloroform, and dichloromethane. From these, combinations of methanol, ethyl acetate, and toluene gave good results in terms of separation and therefore, further trials were initiated for different ratios of methanol, ethyl acetate, and toluene with addition of different modifiers such as glacial acetic acid, ammonia, formic acid, ortho phosphoric acid, and ammonium formate. Band characteristic was improved by addition of acetonitrile to the above mobile phase. However, considerable fronting was observed in ND, and therefore 3M ammonium formate was added to minimize fronting. Finally, the mobile phase

Table 2 – Analytical validation parameters of proposed HPTLC method for simultaneous estimation of ND, MF, and MN.

Analytical parameters	ND	MF	MN
Calibration range[a] (ng/band)	400–2400	100–600	400–2400
Regression equation	4.5482x + 2460.7	7.6661x + 357.79	3.5916x + 1285
Coefficient of determination (r^2)	0.9976	0.996	0.9964
Standard deviation of slope	0.0056	0.0626	0.0527
Confidence limit of slope[b]	4.55–4.56	7.60–7.73	3.58–3.65
Standard deviation of intercept	6.64	16.35	48.73
Confidence limit of intercept[b]	2446.59–2460.32	340.87–374.68	1234.68–1335.47
Limit of detection (ng/band)	9.57	7.03	44.77
Limit of quantification (ng/band)	29.00	21.32	135.69
Precision study			
Repeatability[c]	0.54–1.49	0.37–1.17	0.15–1.14
Interday precision[c] (d 1)	1.06–1.63	0.72–1.56	0.95–1.20
Interday precision[c] (d 2)	1.16–1.61	0.75–1.65	1.09–1.17
Accuracy[c] (%)	98.35–99.76	99.36–99.65	99.16–100.25
Bartlett's test[d](χ2)	0.00019	0.00113	0.003

HPTLC = high performance thin layer chromatography; MF = mometasone furoate; MN = miconazole nitrate; ND = nadifloxacin.
[a] Mean of three determinations.
[b] Confidence interval at 95% confidence level and four degree of freedom.
[c] n = 3 replicates.
[d] Calculated value χ2 less than critical value χ^2(0.05, 4) = 9.488.

consisting of methanol:ethyl acetate:toluene:acetonitrile:3M ammonium formate in water (1:2.5:6.0:0.3:0.2% v/v/v/v) gave a sharp and symmetrical peak. Well defined bands of ND at R_f 0.23 ± 0.02 (1200 ng/band), MF at R_f 0.70 ± 0.02 (300 ng/band), and MN at R_f 0.59 ± 0.05 (1200 ng/band) were obtained when the chamber was saturated with the mobile phase for 20 minutes at room temperature and detection wavelength was 224 nm (Figure 2).

ND, MF, and MN showed a good coefficient of determination in the given concentration range of 400—2400 ng/band for ND and MN and 100—600 ng/band for MN respectively (Table 2). Homoscedasticity of variance was confirmed by Bartlett's test and the response of peak area for all three drugs showed homogenous variance that was exemplified by the χ^2 value less than the tabulated value (Table 2). Limits of detection for ND, MF, and MN were found to be 9.57 ng/band, 7.03 ng/band, and 44.77 ng/band respectively. Limits of quantitation for ND, MF, and MN were found to be 29.00 ng/band, 21.32 ng/band, and 135.69 ng/band respectively indicating good sensitivity of the method.

The precision of the developed method was evaluated by repeatability and intermediate precision, and was expressed as %RSD of peak area. Repeatability and intermediate precision was carried out by performing three replicates of three different concentrations (800 ng, 1600 ng, and 2400 ng for ND and MN, 200 ng, 400 ng, and 600 ng for MF) showed %RSD < 2% (Table 2), indicating acceptable precision in terms of repeatability of peak area measurement and sample application. An accuracy study by standard addition method showed percentage recovery at all three levels in the range of 98.35—100.25%, suggesting suitability and applicability of method for routine drug analysis (Table 2).

The marketed formulation using the developed method, showed three peaks at R_f of 0.20, 0.70, and 0.59 for ND, MF, and MN that was found to be at the same R_f for all three respective standards (Figure 3). The peak purity of ND, MF, and MN in marketed formulations when evaluated by comparing the spectra at peak start, peak apex, and peak end positions of the band (Figures 3A—3C) showed good correlation i.e., r (S,M) and r (M,E) for ND was 0.9993 and 0.9960, for MF 0.9993 and 0.9985,
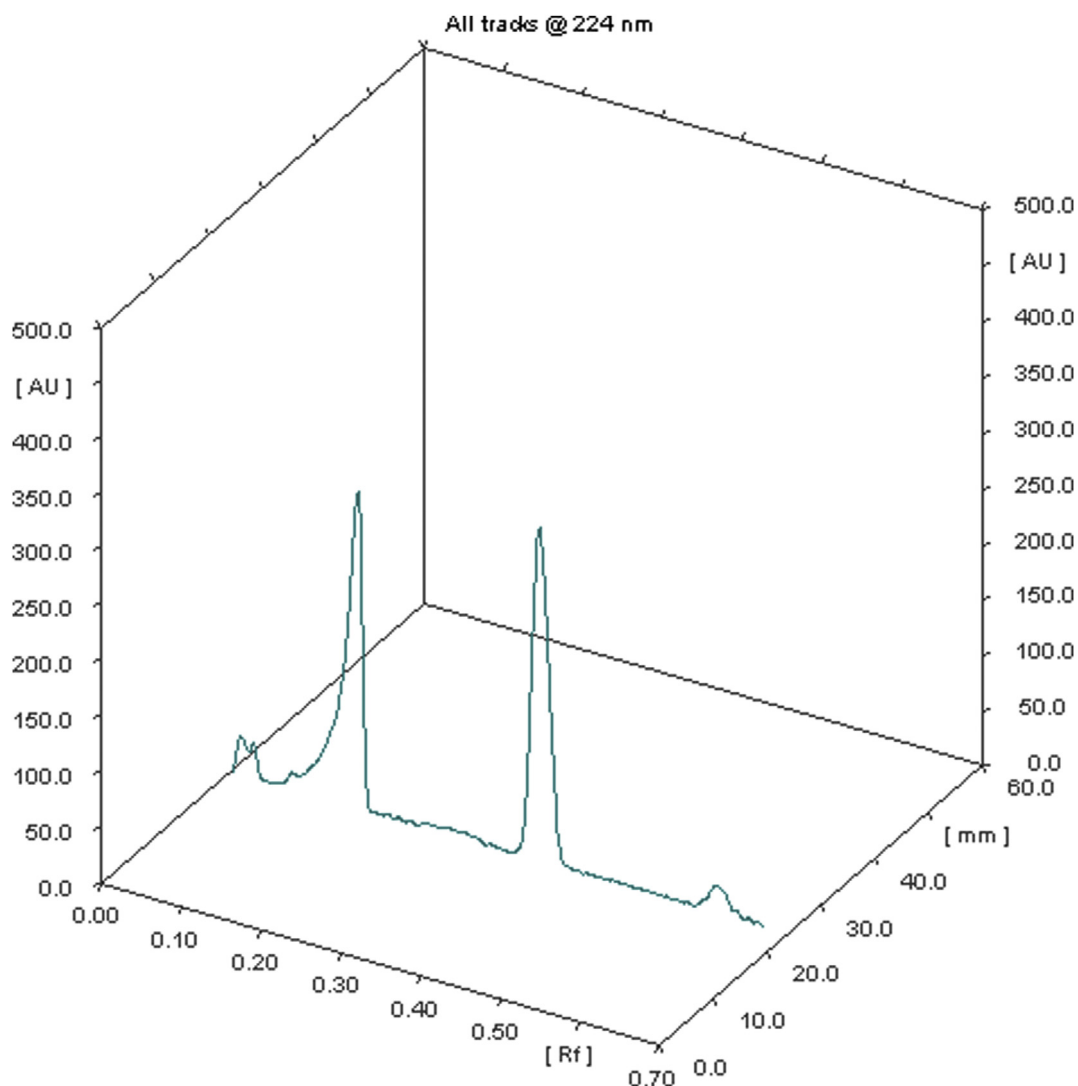
Figure 3 — TLC chromatogram of formulation, showing peaks of ND, MF, and MN. MF = mometasone furoate; MN = miconazole nitrate; ND = nadifloxacin; TLC = thin layer chromatography.
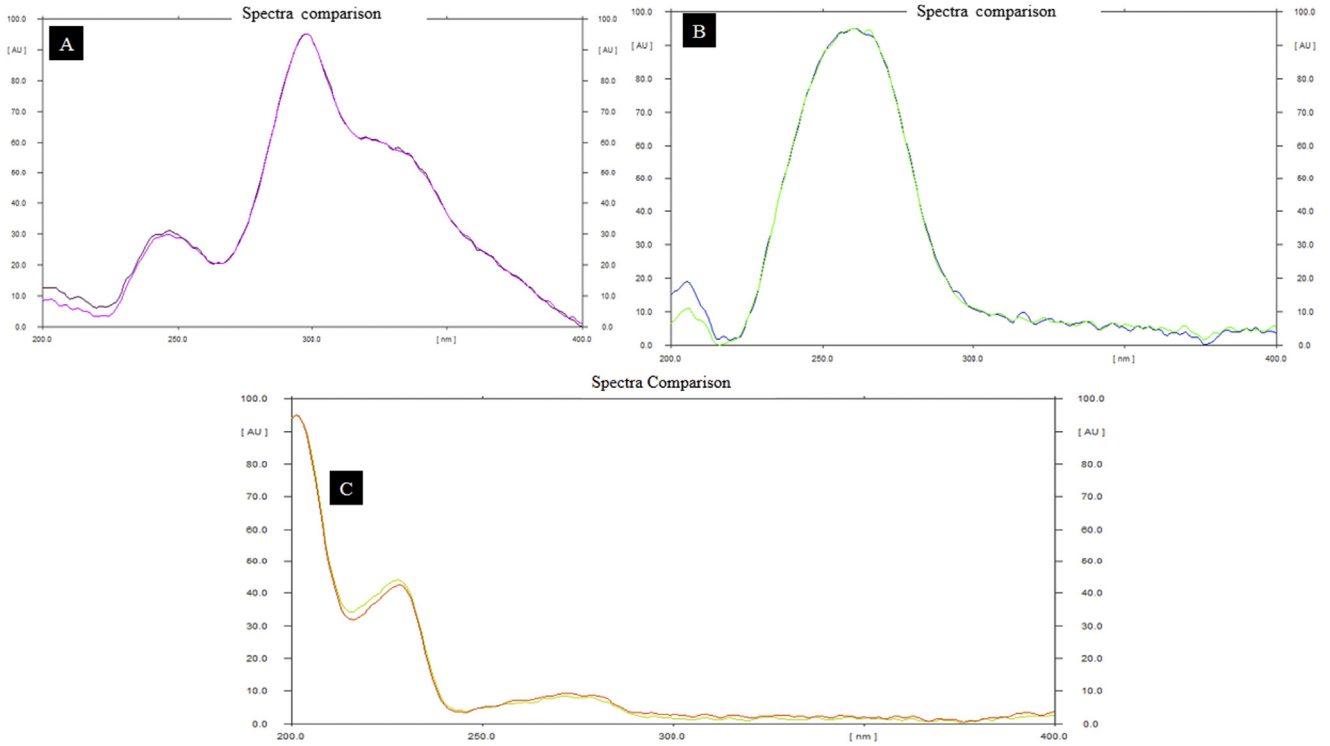
Figure 4 – *In situ* overlaid spectra of samples with standard showing peak purity, ND (A), MF (B), and MN (C).
MF = mometasone furoate; MN = miconazole nitrate; ND = nadifloxacin.
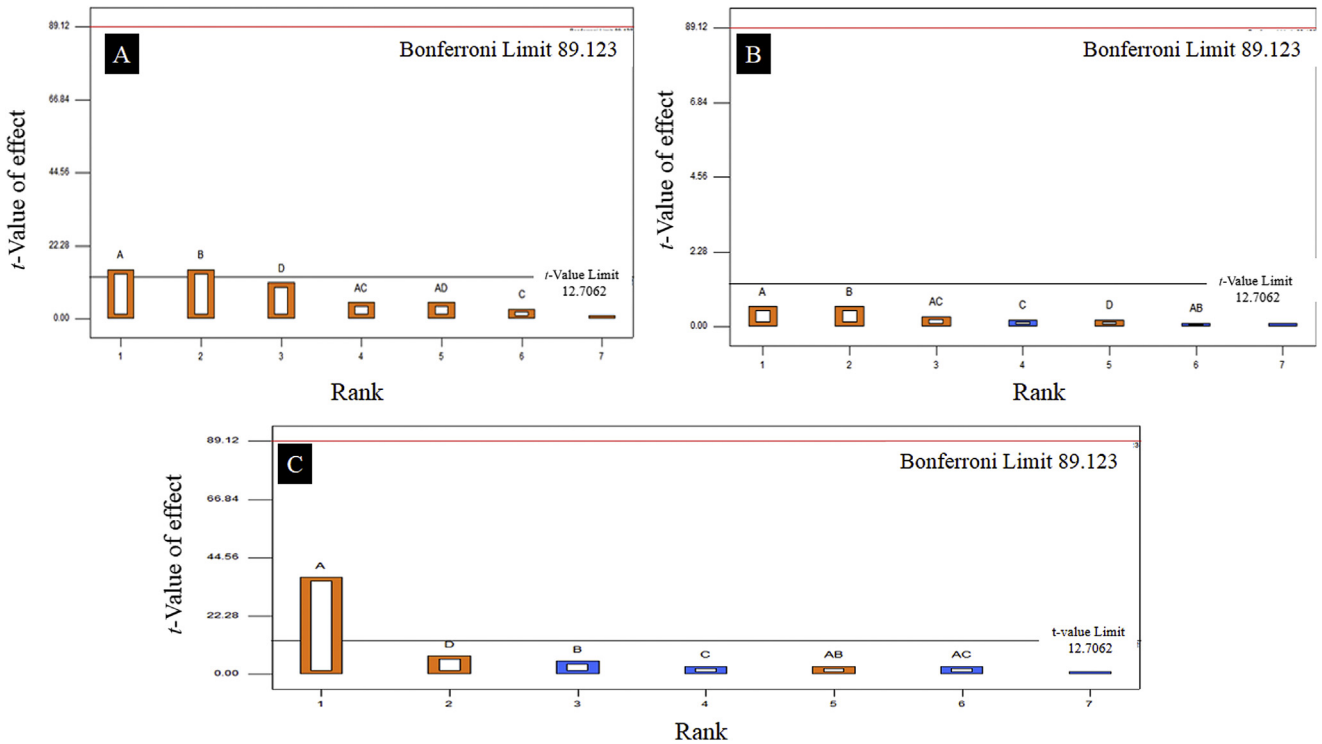

Figure 5 – Pareto chart showing the effect of factors and interaction on R_f values of ND (A), MF (B), and MN (C).
MF = mometasone furoate; MN = miconazole nitrate; ND = nadifloxacin.
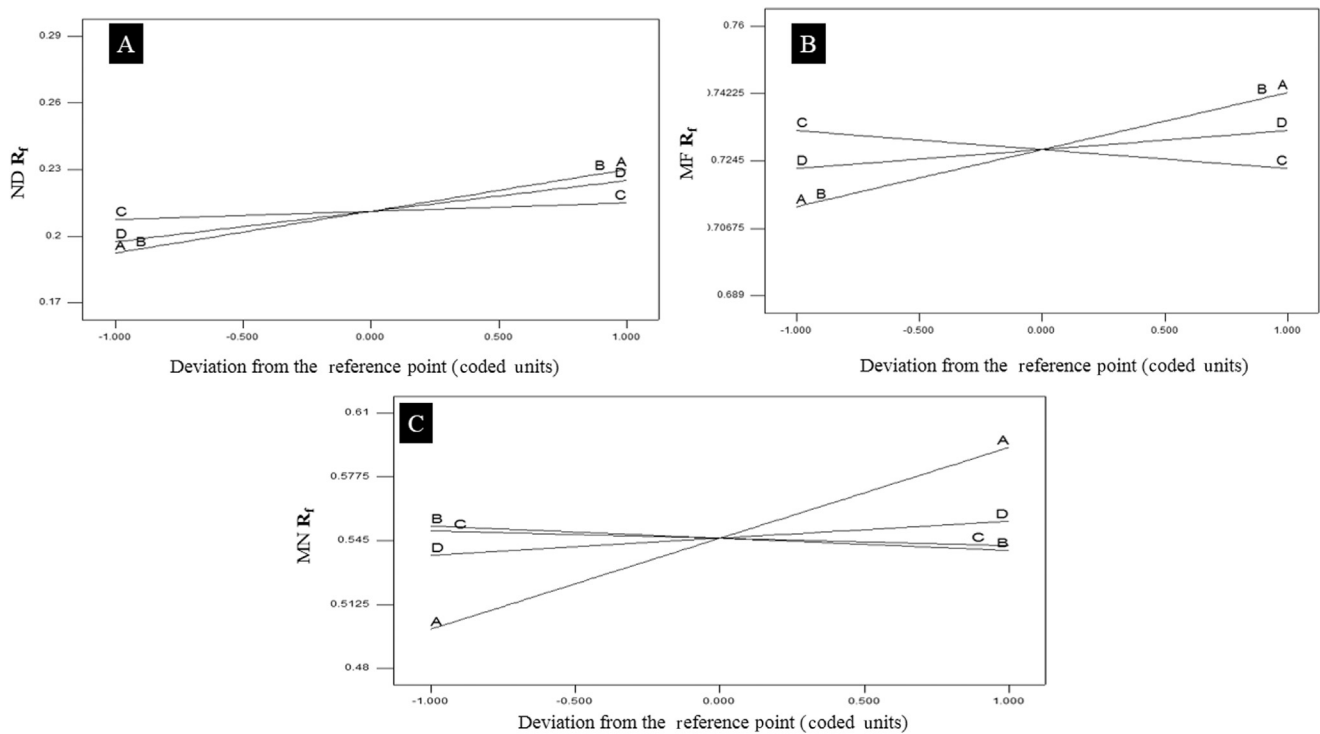
Figure 6 – Perturbation plot showing effect of factors on R_f values of ND (A), MF (B), and MN (C). MF = mometasone furoate; MN = miconazole nitrate; ND = nadifloxacin.
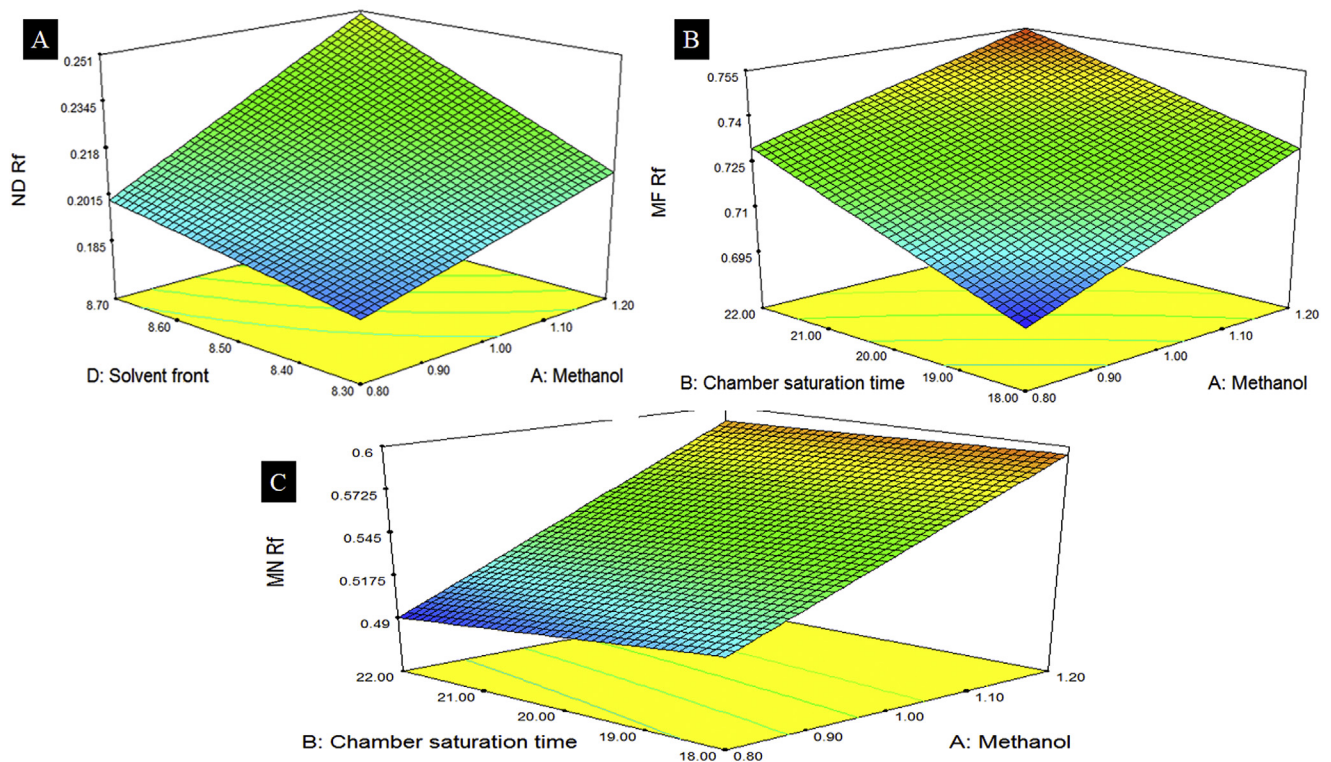

Figure 7 – Three-dimensional response surface plot showing effect of factors on R_f values of ND (A), MF (B), and MN (C). MF = mometasone furoate; MN = miconazole nitrate; ND = nadifloxacin.

Table 3 — Predicted response models and statistical parameters by ANOVA analysis.				
Response (R_f value)	Polynomial equation model for retardation factor	Model p value	% CV	Adequate precision
ND	$0.21 + 0.019*A + 0.019*B + 0.00375*C + 0.014*D + 0.00625$ $*A*C + 0.00625*A*D$	0.0746	1.67	**33.26**
MF	$0.73 + 0.015*A + 0.015*B - 0.0050*C + 0.005*D - 0.0025*A*B + 0.0075*A*C$	0.1951	0.97	**11.34**
MN	$0.55 + 0.046*A - 0.0625*B - 0.00375*C + 0.00875*D + 0.00375*A*B$ $-0.00375*A*C$	0.0489	0.65	**39.31**

Bold values signify the adequate precision value must be greater than 4 indicates greater signal to noise ratio.
ANOVA = analysis of variance; CV = coefficient of variance; MF = mometasone furoate; MN = miconazole nitrate; ND: nadifloxacin.

and for MN 0.9993 and 0.9981. Therefore, the method was found to be specific in the presence of various excipients (Figures 4A—4C).

All robustness testing runs were performed in a randomized order to minimize the effects of uncontrolled factors that may introduce bias to the response. Graphical interpretation in form of response surfaces and perturbation plots showed the correlation of the effect of the factors on the retention factor of each drug. Perturbation plots reveal the change in response from its nominal value with all other factors held constant at a reference point, and steepest slope or curvature indicates sensitiveness to specific factors.

The Pareto chart is useful for checking the significance of factors, where effects above the Bonferroni Limit are almost certainly significant; effects above the t-value limit are possibly significant and effects below the t-value limit are not likely to be significant. The Pareto chart for all the three drugs reveals that volume of methanol in mobile phase had important effects on retention factor of drugs, in decreasing order: for ND, A > B > D > AC > AD; for MF, A > B > AC > C > D; and for MN, A > D > B > C = AB = AC, as shown in Figures 5A—5C.

Perturbation plots indicated that small variation in volume of methanol and chamber saturation time had important effects but did not produce any significant effect on retention factor except MN as shown in Figures 6A—6C. As can be seen from the three-dimensional response surface plots, an increase in methanol content of mobile phase produced an increase in R_f of all three drugs as shown in Figures 7A—7C.

The model was validated by analysis of variance (ANOVA) using Design Expert software (Table 3). The equation in terms of coded factors can be used to make predictions regarding the response for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. Model p value > 0.05

indicates that factors had nonsignificant effect on response resulting in a robust method. Adequate precision defined as a signal-to-noise ratio > 4 is desirable, and the obtained ratio for all the three drugs indicated an adequate signal (Table 4). The low standard deviation [% coefficient of variance (CV)] and adequate precision, indicates a good relationship between the experimental data and those of the fitted models.

The cream formulation, Bactimax (7.5g), containing ND (1%), MF (0.1%), and MN (2%) when analyzed in triplicate using the developed HPTLC method showed good recovery where percentage amount for all the drugs were within the range of 97.05%—99.73% with %RSD < 2 (Table 4) indicating that the method can be applicable in routine quality control testing of the cream.

The developed method was found to be novel, simple, accurate, precise, specific, and reproducible for the simultaneous estimation of ND, MF, and MN in cream formulations. Moreover, the major advantage of developed HPTLC method is that several samples can be run simultaneously using a small amount of mobile phase unlike HPLC, thus lowering analysis time by high sample throughput and cost per analysis. The application of FFD on robustness was to study simultaneous variation of effects on responses. Methanol content in mobile phase appeared to have possibly significant effects on response of MN and non significant effects on response of ND and MF in robustness study compared with other factors and therefore it was important to be carefully controlled. It is concluded that the use of experimental design and response surface methodology is a flexible procedure, able to reduce the number of the needed experiments for the robustness study of HPTLC method. The method was found to be repeatable and suitable for routine quality control and combined dosage form analysis.

Conflicts of interest

All authors have no conflicts of interest.

Acknowledgments

Table 4 — Analysis of pharmaceutical formulation.			
Drug	Label claim (% w/w)	% Amount of drug found[a]	% RSD
ND	1% w/w	99.73	0.64
MF	0.1% w/w	97.05	1.27
MN	2% w/w	99.67	0.35

%RSD = relative standard deviation; FFD = fractional factorial design; MF = mometasone furoate; MN = miconazole nitrate; ND = nadifloxacin.
[a] Mean of three determinations.

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